MECHANISM OF TRANSCRIPTION REGULATION AND STABILITY OF THE QUORUM SENSING PROTEIN TRAR

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MECHANISM OF TRANSCRIPTION REGULATION AND STABILITY OF THE QUORUM SENSING PROTEIN TRAR

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TraR is a transcription activator of the quorum sensing system in the plant pathogen *Agrobacterium tumefaciens.* This protein is composed of two domains: amino-terminal domain (NTD) which is responsible for binding the autoinducer OOHL and for dimerization and the carboxyl-terminal domain (CTD) which contributes less extensively to dimerization and binds to specific sequences in the DNA called *tra* boxes. Despite being one of the most studied proteins from the LuxR family, the mechanism of transcription activation and stability of TraR are not well understood. In the first part of this work, we construct mutations in amino acids in the NTD of TraR to find the ones involved in the contact with RNA polymerase. Two patches of amino acids were found. One of them is composed of amino acids that are important for class I and II promoters. Therefore, those amino acids might contact the alpha-CTD subunit of the RNA polymerase. The other patch has amino acids important only for class II promoters, indicating that they might contact alpha or sigma subunits of the RNA polymerase. The second part of this study shows that the CTD of TraR contains amino acids that destabilize the protein. The results indicate that amino acids that are exposed in monomer TraR, but

buried in the dimer protein, can provide protease recognition motifs. It was also shown that amino acids involved in RNA polymerase and DNA contacts, can also destabilize TraR. These results suggest that when bound to DNA or RNA polymerase, this amino acids would not be available for proteolysis. In the third part of this study, the effect of the antiactivator protein, TraM, in the stability of TraR was studied. The results show that TraR is degraded after antiactivation and that the levels of TraM do not change. Mutants that block antiactivation also block TraR proteolysis. The results suggest that TraM is an adaptor protein which delivers TraR to proteases, increasing its degradation rate.

BIOGRAPHICAL SKETCH

Esther Dantas Costa was born on January 29th 1982 in Viçosa, Minas Gerais in the southeastern part of Brazil. At the age of 3, she moved to Paranã, Tocantins, a beautiful city in the north of Brazil, with two big rivers, only one public school, no pavement, electricity for only a few hours a day and lots of snakes. She lived there for almost ten years. This made for a very fun childhood for her and for sure had a big influence in her character. In 1994, she moved back to Viçosa where she graduated from Equipe high school. In 1999, she was admitted to the Universidade Federal de Viçosa, majoring in Food Engineering in 2004. During four years of her undergraduate studies, she worked at the Food microbiology Lab, where she received a scientific initiation fellowship from the Brazilian government (CNPq). In 2004 she started the Master's program in Agricultural Microbiology also at Universidade Federal de Viçosa with an award from CNPq. She then decided to study abroad and won a fellowship to come to the United States to pursue a PhD. She received her M. Sc. in 2006 and came to Cornell University in the same year where she joined the laboratory of Dr. Stephen C. Winans in August of 2006.

Para os meus queridos pais, Denise e Simião, e avós, Esther e Sebastião

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CHAPTER 1

Introduction

1.1. Cell-cell communication in bacteria

In the past it was believed that bacteria worked as single cells independent of one another, and that the phenotypes expressed by each cell did not depend on the other bacteria present in their environment. In the 1960s and 1970s however, studies on *Streptococcus pneumonia* and *Vibrio fischeri* revealed that certain bacterial traits were expressed only after a particular bacterial cell density was reached (Nealson *et al.*, 1970; Tomasz, 1965). This suggested that the bacteria had some means to communicate with each other, and were not acting in isolation.

Within a few years, pheromone-like extracellular products involved in this cell-cell communication were identified (Eberhard *et al.*, 1981; Lazazzera and Grossman, 1998). This opened the door for the investigation of the mechanisms and processes behind this type of communication.

For many years, this communication phenomenon was believed to only play a role in a limited number of organisms. However, in the 1990s several researchers observed similar phenomenon in a wide range of different bacterial species, including in *Agrobacterium tumefacies* (Atkinson *et al.*, 1999; Beck von Bodman and Farrand, 1995; Fuqua and Greenberg, 1998; Fuqua *et al.*, 1994; Gray *et al.*, 1996; Latifi *et al.*, 1995; Lazazzera and

Grossman, 1998; Lewenza *et al.*, 1999; Lindum *et al.*, 1998; Piper *et al.*, 1993; Puskas *et al.*, 1997; von Bodman *et al.*, 1998).

In 1994, the term “quorum sensing” was coined to describe this communication, reflecting the idea that bacterial cells carry out certain behaviors only if and when they reach some minimum cell population density, or “quorum” (Fuqua *et al.*, 1994). These regulatory systems, by using diffusible pheromones, provide a mechanism for bacteria to gauge their population densities, and to initiate gene expression only at high cell densities. Since then, the term “quorum sensing” (QS) has been widely adopted to describe cell-cell communication in bacteria, yeast, insects, and vertebrates.

During the 1990s, the accelerated pace of research on quorum sensing enabled much improved understanding of the process. This in turn aroused the interest of the scientific community to the many possible applications of QS. These potential applications include uses in the production of biochemicals, tissue engineering and mixed-species fermentations (Choudhary and Schmidt-Dannert, 2010). Use of QS-inhibitors also has the potential to control the expression of virulence genes in pathogenic bacteria, thereby preventing disease (Ni *et al.*, 2009; Njoroge and Sperandio, 2009).

1.2. *Agrobacterium tumefaciens*

This dissertation will mainly focus on the bacterium *A. tumefaciens* where quorum sensing has been seen to play a role in the conjugation and replication of the tumor-inducing (Ti) plasmid (Fuqua and Winans, 1994; Li and Farrand, 2000; Pappas and Winans, 2003b).

A. tumefaciens belongs to the *Rhizobiaceae* family of the alphaproteobacteria group, which also includes nitrogen-fixing symbionts of legumes (Goodner et al., 2001; Slater et al., 2009; Wood et al., 2001). *A. tumefaciens* causes crown gall tumor in plants (Smith and Townsend, 1907), and is a problem in the agricultural industry since infected plants show decreased productivity (Escobar and Dandekar, 2003). *A. tumefaciens* has the unique ability to modify the genome of host plants (Chilton et al., 1977; Winans, 1992). This bacterium is used as a model organism for horizontal gene transfer, type IV secretion, plant-bacteria signaling, and quorum sensing (Farrand et al., 2002; Zhu and Winans, 2001).

The genome of *A. tumefaciens* strain C58 (biovar I) was sequenced in 2001 and is composed of two chromosomes, one linear and one circular, and two plasmids (Goodner et al., 2001; Wood et al., 2001). Other two genomes of this genus were sequenced in 2009, from strain *A. radiobacter* K84 (biovar II) and strain *A. vitis* S4 (biovar III) (Slater et al., 2009). The Tumor-inducing (Ti) plasmid from biovar I is going to be the focus of this section because it

contains both the tumor-inducing and the quorum sensing genes, which are of particular interest in the context of this work.

The Ti plasmid is approximately 200 Kb in length and contains most of the genes required for crown gall formation. A set of genes denoted *vir* genes direct the processing and transfer a piece of oncogenic DNA (T-DNA) to genome of the host plant. This T-DNA is then integrated into a chromosome of the host. Ti plasmids from different strains of *A. tumefaciens* are classified by opine type. The octopine (including A6 and R10 strains) and the nonpaline Ti plasmids (including C58 strain) are the most studied ones. The genes encoded by pTiR10 can be viewed in Figure 1.1 (Zhu et al., 2000). The main regions of this plasmid are: T-DNA region which has 25 bp unique border regions, not present any place else in the genome; virulence (*vir*) region which is responsible for processing and transferring the T-DNA from the bacteria to the plant cell; replication and partitioning (*rep*) region; and the *tra* and *trb loci*, which are involved in the conjugation of the plasmid. The plasmid also encodes genes responsible for the uptake and the catabolism of opines, which allows the bacteria to use the opines produced by the host plant after infection. Some opines are derivatized amino acids, while others are novel carbohydrates (Oger and Farrand, 2001; Zhu *et al.*, 2000).

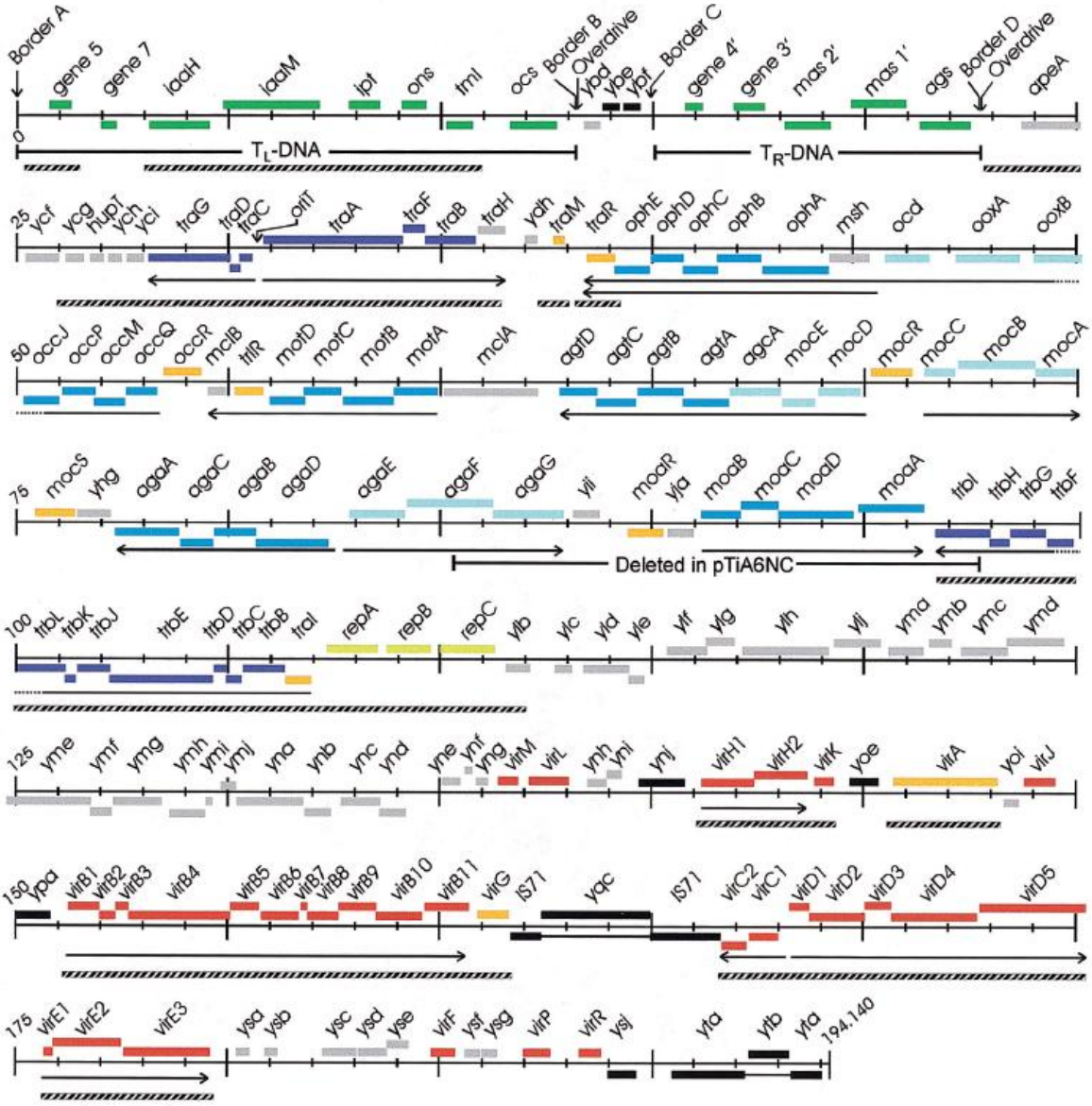

Figure 1.1. The genes of the octopine-type Ti plasmid and their organization. The genes shown above the scale are transcribed from left to right while the ones transcribed from right to left are below the scale. Green bars indicate genes that are transferred to plant cells. Red bars indicate genes in the *vir* regulon, regulated by VirA and VirG, and required for T-strand processing and transfer. Purple bars indicate genes required for conjugation of the Ti plasmid. Light green bars indicate genes required for replication and partitioning of the Ti plasmid. Dark blue bars indicate genes encoding opine permeases. Light blue bars indicate genes involved in opine catabolism. Orange bars indicate regulatory genes. Black bars indicate suspected IS elements and grey bars indicate ORFs of miscellaneous or unknown function. Figure from Zhu et al. (Zhu *et al.*, 2000).

Exudates from the host plants, including phenolic compounds, such as acetosyringone, sugars and acidity, are detected by periplasmic sugar-binding protein called ChvE and by a two-component signal transduction system composed of two proteins encoded in the Ti plasmid, VirA and VirG (Huang *et al.*, 1990; Winans *et al.*, 1986; Winans *et al.*, 1994). Upon detection of the signals, a phosphorylation cascade is activated where VirA, a transmembrane histidine kinase, transfers a phosphoryl group to VirG, which is the response regulator. Phosphorylated VirG can activate transcription of other *vir* genes (Gelvin, 2006, 2009).

The *vir* regulon has approximately 30 genes, most of which encode proteins necessary for the transfer and integration of the T-DNA into the plant cell nucleus. The T-DNA is processed by VirD2, which forms a complex with the T-DNA by binding to its 5' end (Stachel *et al.*, 1986). VirE2 binds to the entire surface of the single stranded T-DNA (Citovsky *et al.*, 1997). The complex VirD2-T-DNA-VirE2 is transferred to the plant using a type IV secretion system formed by proteins of the *virB* operon and VirD4 (Cascales and Christie, 2003; Gelvin, 2009). VirD2 and VirE2 have nuclear localization signals, which are recognized by the host and the protein-DNA complex is translocated to the nucleus (Ballas and Citovsky, 1997; Citovsky *et al.*, 1992; Howard *et al.*, 1992). Once in the nucleus, the T-DNA is integrated randomly into the plant chromosome by illegitimate recombination (Mayerhofer *et al.*, 1991).

The T-DNA contains approximately 15 genes, some of which play a variety of roles in uncontrolled cell proliferation, and the formation of the tumors characteristic of crown gall disease (Escobar and Dandekar, 2003). Other transferred genes direct the production of novel compounds called opines, which diffuse out of the plant, and serve as a nutrient for the bacteria.

The opines produced by the host plant not only serve as nutrients for the bacteria, but also provide signals for the activation of the genes related to quorum sensing. Quorum sensing in this bacterium is responsible for the conjugation and replication of the Ti plasmid (Fuqua and Winans, 1996b; Piper *et al.*, 1999). Quorum sensing in *A. tumefaciens* is described in detail in section 1.3.

1.3. Quorum sensing in *A. tumefaciens*

During quorum sensing, bacteria communicate with each other and coordinate their behavior by responding to chemical molecules, also known as signal molecules, pheromones or autoinducers (Fuqua and Greenberg, 2002; Waters and Bassler, 2005; Whitehead *et al.*, 2001). These signals are used by a bacterium to monitor the cell density in its neighborhood and this information is then used to guide the bacteria to behave in a particular way. The signal molecules vary according to the bacterial group. Acyhomoserine lactones (AHLs) are normally the mediators of communication in Gram negative bacteria while for Gram positive species, small peptides are involved in

signaling (Fuqua *et al.*, 2001; Lazazzera and Grossman, 1998; Platt and Fuqua, 2010; Waters and Bassler, 2005; Whitehead *et al.*, 2001). In addition, a molecule called autoinducer 2 (AI-2) is commonly believed to be a signal for many groups of bacteria, however there is still some controversy surrounding this issue (Bassler *et al.*, 1997; Fuqua and Greenberg, 2002; Ng *et al.*, 2011; Rezzonico and Duffy, 2008). New signals and signaling systems are being discovered every year and it is expected that new families of signals will be discovered in the coming years. Bacterial quorum sensing signals already described are summarized in Figure 1.2 and include gamma-butyrolactones, fatty acids, fatty acid esters, quinolones and CAI-1 family (Winans, 2011).

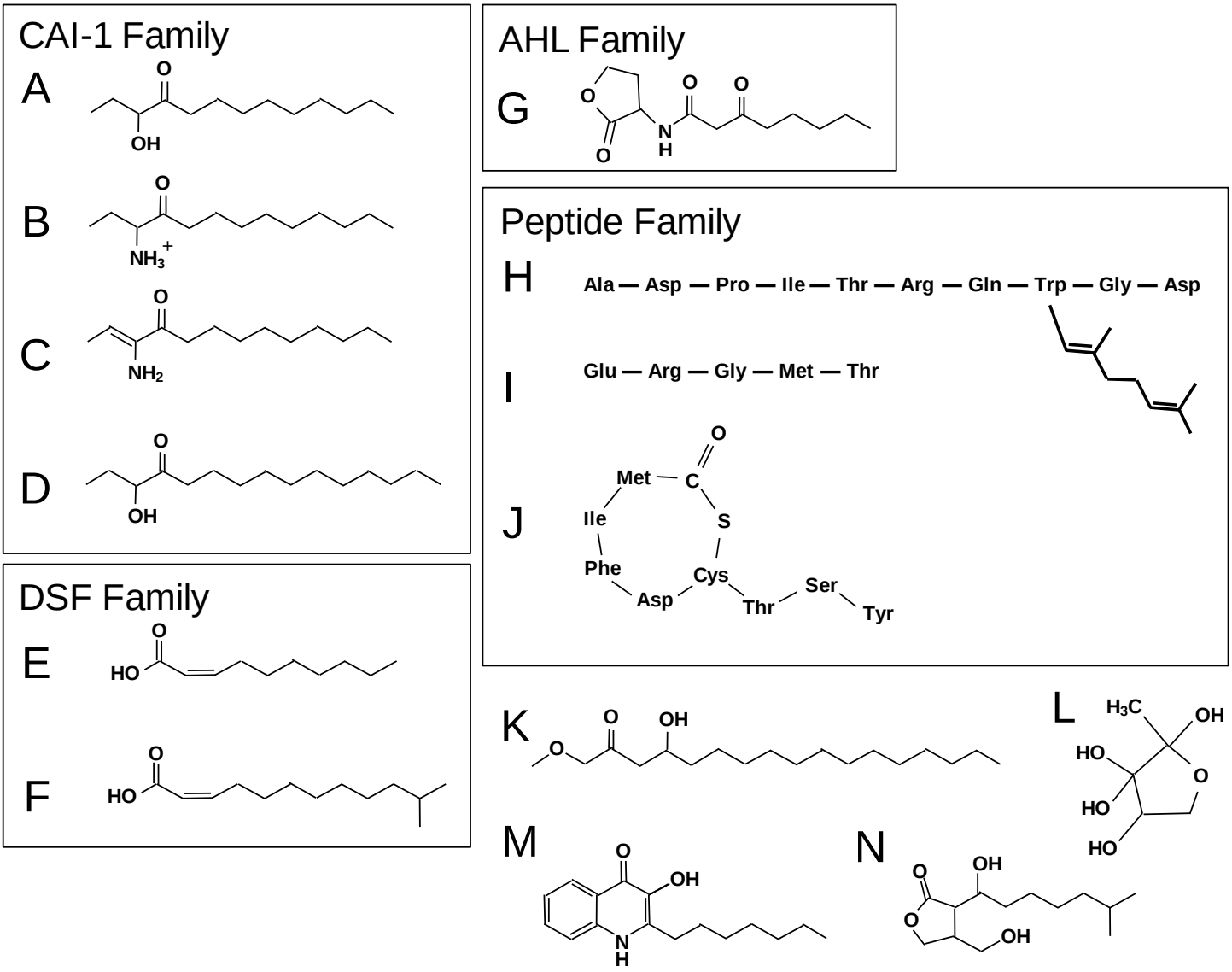

Figure 1.2. Signal molecules used in bacterial QS signaling. (A) CAI-1 (3-hydroxytridecan-4-one, *V. cholerae*). (B) Am-CAI-1; (3-aminotridecan-4-one, *V. cholerae*). (C) Ea-CAI-1 (3-enaminotridecan-4-one, *V. cholerae*). (D) LAI-1 (3-hydroxypentadecan-4-one, *Legionella pneumophila*). (E) cis-decenoic acid, (*P. aeruginosa*). (F) DSF (11-methyl-cis-dodecenoic acid, *X. campestris*). (G) OOHL (3-oxo-octanoylhomoserine lactone, *A. tumefaciens*). (H) ComX (isoprenylated peptide, *B. subtilis*); (I) CSF (unmodified pentapeptide, *B. subtilis*). (J) AIP-1 (thiolactone peptide, *S. aureus*); (K) PAME (palmitic acid methyl ester, *R. solanacearum*). (L) A1-2 ((2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran, ubiquitous). (M) PQS (2-heptyl-3-hydroxy-4-quinolone, *P. aeruginosa*). (N) A-factor (*Streptomyces* spp.). Figure modified from (Winans, 2011).

Quorum sensing was described for the first time in the marine bacteria *Vibrio fischeri*, which is a symbiont of various marine animals (Nealson *et al.*, 1970; Nealson and Hastings, 1979). The quorum sensing system in this bacterium became the paradigm and it is illustrated in Figure 1.3. In this system, a protein called LuxI (known as the autoinducer synthase) produces the signal molecule N-3-oxohexanoyl-L-homoserine-lactone (OHHL) (Eberhard *et al.*, 1981). OHHL diffuses passively from the cells into the surroundings. The LuxR protein is an OHHL receptor and OHHL-dependent transcription factor. The presence of OHHL enables LuxR to form a dimer, which in turn binds to the luciferase promoter region and activates transcription of genes responsible for bioluminescence (Antunes *et al.*, 2007; Pappas *et al.*, 2004; Waters and Bassler, 2005; Whitehead *et al.*, 2001). The amino terminal domain of LuxR binds to OHHL with a dissociation constant of 100 nM (Urbanowski *et al.*, 2004) and mediates dimerization while the C-terminal domain binds to a 20-base-pair inverted repeat termed *lux* box centered 42.5 nucleotides upstream of the transcription start site of the *lux* operon (Antunes *et al.*, 2008; Urbanowski *et al.*, 2004).

At low cell densities, the concentration of OHHL is not enough to activate LuxR so the communication chain is not activated. At a sufficiently high population, OHHL molecules accumulate such that their efflux is balanced by an influx. OHHL then binds to LuxR, converting it to an active form. In this way, the bacterium learns of, and responds to, the presence of a high concentration of nearby bacteria of the same species.

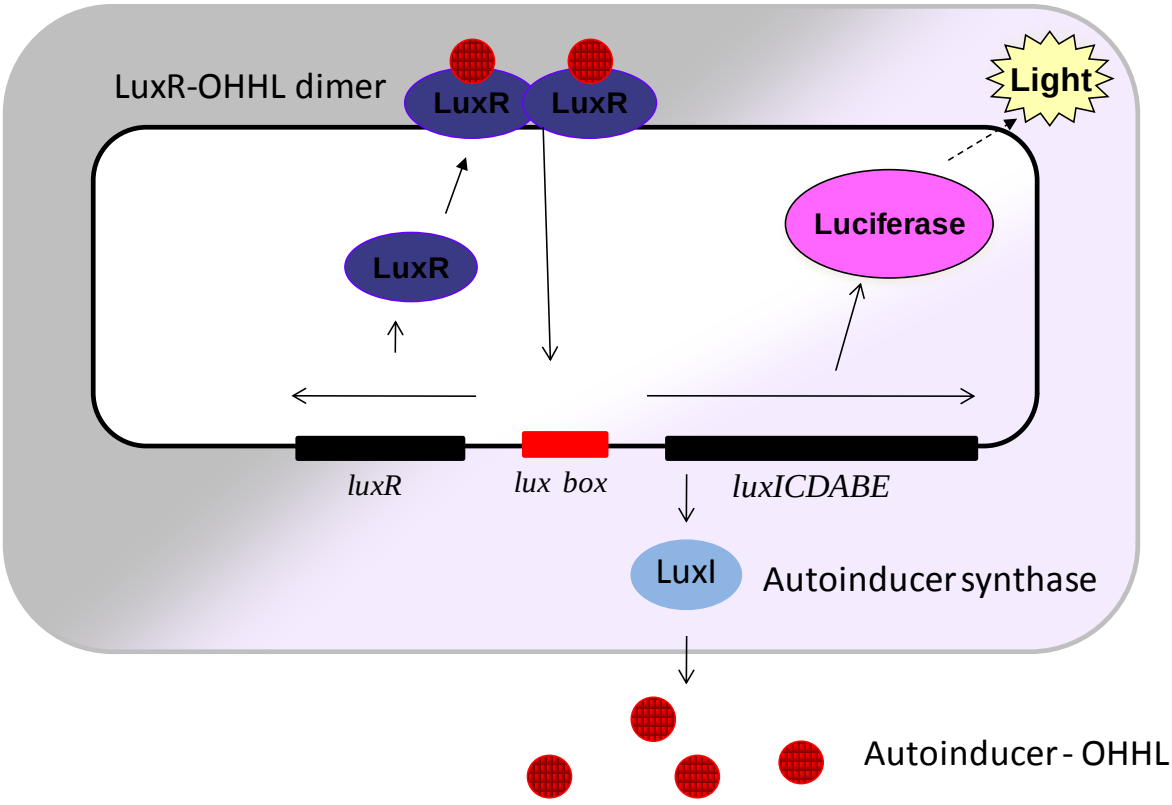

Figure 1.3. The Quorum sensing mechanism in *V. fischeri*. At low cell density, the autoinducer synthase LuxI synthesizes N-3-oxohectanoyl-L-homoserine lactone (OHHL - red circles) which diffuses across the membrane. When a high cell density is reached, the OHHL accumulates inside the cells, binds to the transcription activator LuxR. LuxR-OHHL complexes form dimers which bind to target DNA, called *lux* boxes, activating transcription of *luxI*, the autoinducer synthase, and l*uxCDABE* which are responsible for bioluminescence.

Proteins from the LuxR/LuxI family are present in many proteobacteria, including in *A. tumefaciens,* and they regulate many different phenotypes, including biofilm formation, pathogenesis, and production of secondary metabolites (Fuqua *et al.*, 2001; Miller and Bassler, 2001; Waters and Bassler, 2005; Whitehead *et al.*, 2001). In these systems, the signal molecule is produced by a synthase that resembles LuxI and is detected by a protein that resembles LuxR. In most cases, the signal molecule converts its receptor to an active form. However, there are some exceptions to this. In some cases, the signal converts the receptor to an inactive form that dissociates from the DNA (Tsai and Winans, 2010). Proteins of this family include the EsaR of *Pantoea stewartii,* ExpR of *Pectobacterium carotovorum* and *Erwinia chrysanthemi*, SmaR of *Serratia* sp*.*, YenR of *Yersinia enterocolitica,* PsyR and AhlRI of *Pseudomonas syringae* (Carlier *et al.*, 2009; Castang *et al.*, 2006; Cui *et al.*, 2005; Fineran *et al.*, 2005; Minogue *et al.*, 2002; Minogue *et al.*, 2005; Schu *et al.*, 2009; Sjoblom *et al.*, 2006; Tsai and Winans, 2010, 2011). Some of the characteristics of these proteins reviewed by Tsai and Winans (Tsai and Winans, 2010) are:

- Bind preferentially to OHHL (SmaR of *P. Carotovorum* is an exception and preferentially binds BHL
- Do not have any effect on the synthase genes, differently from the LuxR-type proteins which normally activate expression of their cognate AHL synthase genes

- Can function as repressors (although some can also activate certain promoters)
- Are encoded by genes that are adjacent and convergently transcribed from their cognate AHL synthase genes.

Another variation is the so called orphan receptors, which are proteins from the LuxR family that are encoded, but do not have a cognate synthase coupled to them. These receptors are believed to work independently of the signal molecule producer and are believed to be able to recognize AHLs or other small molecules present in the environment (Lee *et al.*, 2006; Patankar and Gonzalez, 2009; Subramoni and Venturi, 2009). *Salmonella* and *E. coli* encode a LuxR homologue called SdiA, but they do not synthesize AHLs. SdiA can detect AHLs produced by other species of bacteria, being a good example of an orphan receptor protein (Dyszel *et al.*, 2010; Noel *et al.*, 2010; Soares and Ahmer, 2011; Sperandio, 2010).

In the case of *Agrobacterium tumefaciens,* a protein called TraI synthesizes N-3-oxooctanoyl-L-homoserine lactone (OOHL) (Figure 1.2) which is detected by a protein called TraR, which is a transcription activator. These two proteins are encoded on the Ti plasmid.

Opines are necessary for the transcription of TraR, and for this reason the quorum sensing system is only activated in the tumor environment, where these molecules are present in abundance. One such opine, octopine, switches the transcription activator OccR from an inactive to an active state. In

the latter state, OccR activates transcription of the *occQ* operon, which includes proteins involved in the uptake and catabolism of opines and TraR (Akakura and Winans, 2002; Fuqua and Winans, 1996b; Habeeb *et al.*, 1991). TraR is not only controlled by the presence of opines, but also in a post-transcription level by OOHL, and by the antiactivator proteins TraM and TrlR (see section 1.5 for more details).

TraR and TraI control the replication and conjugation of the Ti plasmid. One of the genes in the conjugation (*tra)* operon is TraI, which is the OOHL synthase and is positively regulated by TraR, creating a positive feedback loop, also observed in the *V. fischeri* and other bacteria (Fuqua and Winans, 1994; Whitehead *et al.*, 2001). Figure 1.4 presents a model of the regulation of the quorum sensing system in *A. tumefaciens.* The activation of genes involved in conjugation and replication is discussed in more detail in section 1.4.

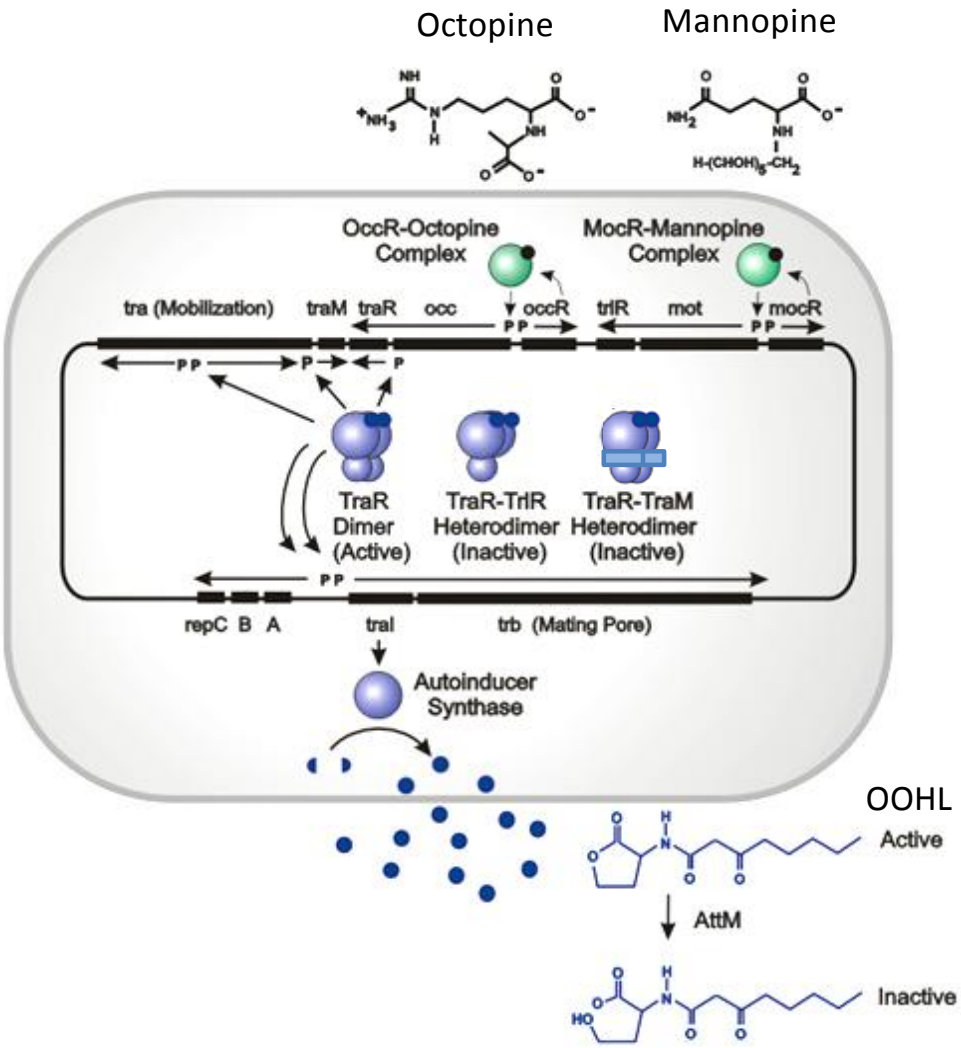

Figure 1.4. The TraR-TraI regulon in the octopine-type Ti plasmid. The octopine produced by the host plants acts as a signal that activates the regulator protein OccR. OccR-octopine complex activates the transcription of *traR*. TraR binds to OOHL, which is the quorum sensing signal produced by TraI. TraR-OOHL binds as a dimer to specific sequences of DNA called *tra* boxes, triggering the transcription of genes responsible for conjugation (*tra* and *trb*) and replication (*rep*) of the Ti plasmid. Another gene regulated by TraR is *traM*, which encodes an antiactivator protein which is discussed in detail below. TraR-OOHL complexes can also be inactivated by TrlR, which is regulated by the transcription activator MocR in the presence of mannopine. AttM is a lactonase which inactivates AHLs, such as OOHL, by opening the lactone ring.

The crystal structure of TraR was published by two groups in 2002 (Vannini *et al.*, 2002; Zhang *et al.*, 2002). The crystal structure shows TraR complexed to OOHL and *tra* box DNA (Figure 1.5). The OOHL is engulfed by the protein in a hydrophobic pocket having no contact with the solvent. The protein binds to DNA as a dimer, and both domains contribute for dimerization. Each monomer of TraR has two domains: the amino terminal domain (NTD) which binds tightly to one molecule of OOHL and the carboxy terminal domain (CTD) which binds to DNA. The NTD and CTD of TraR are linked by a flexible, twelve amino acid long linker (Zhang *et al.*, 2002). The details of the binding between TraR and OOHL and dimerization are discussed in section 1.5 because they are related to the regulation of the levels of TraR in the bacterial cells.

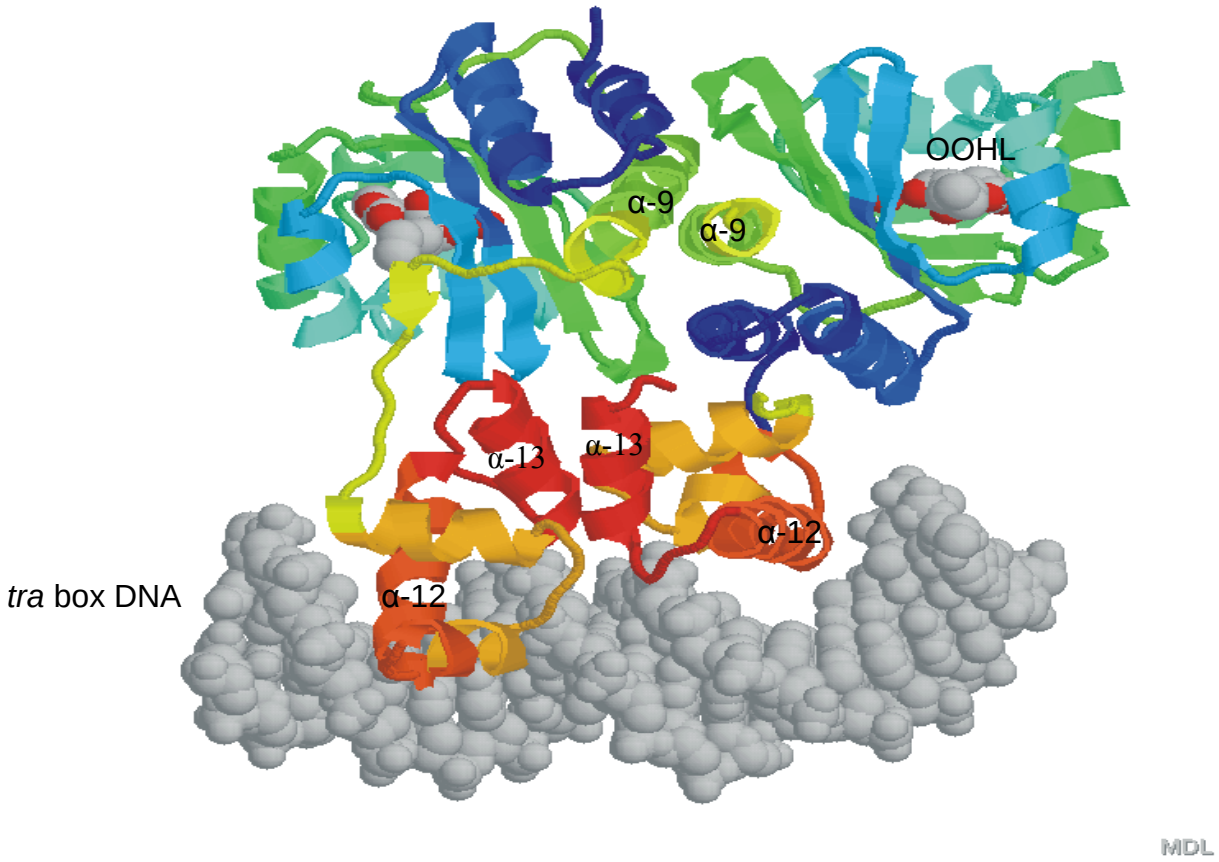

Figure 1.5. Crystal Structure of a TraR dimer complexed to OOHL and bound to its target DNA, *tra* box (pdb code: 1L3L). The NTD of TraR, represented by the blue and green helices, has the OOHL binding domain, indicated on the right monomer and the dimerization domain (helices α-9). The CTD of TraR, represented by the orange and red helices, binds DNA using a helix-turn-helix motif to specific sequences of DNA called *tra* boxes. The DNA recognition helix, α-12, is indicated (Zhang *et al.*, 2002).

1.4. Transcription activation by TraR

As discussed above, TraR activates Ti plasmid genes responsible for conjugative transfer (*tra* and *trb*) and vegetative replication (*rep*) (Cho *et al.*, 2009; Fuqua and Winans, 1994; Li and Farrand, 2000; Pappas and Winans, 2003b). TraR binds using a helix-turn-helix (HTH) motif to 18-nucleotide palindromic DNA sequences called *tra* boxes. Each subunit of the dimer binds half of the *tra* box (Figure 1.6). The contributions of each nucleotide of the consensus *tra box* and of each amino acid from the HTH motif were evaluated, and both specific and non-specific interactions between TraR and DNA were found. The analysis also showed the presence of a sequence in the center of the tra box that does not contact TraR but which is nonetheless important for TraR-DNA affinity, possibly by facilitating bending and flexibility of the DNA (White and Winans, 2007). Some *tra boxes* are centered -43.5 nucleotides upstream of the transcription start site; such promoters are referred to as Class II promoters. Other *tra* boxes bind to a site centered 65.5 nucleotides upstream and are referred to as Class I promoters. This classification was first used for the catabolic repressor protein (CRP) of *E. coli* which is a model system for transcription activation in class I and II promoters (Busby and Ebright, 1999).

TraR regulates a total of seven promoters by binding to specific sites at the DNA called *tra* box I, *tra* box II, *tra* box III and *tra* box IV. Two of the promoters are classified as class I promoters and five as class II promoters (Figure 1.6). The operons controlled by TraR are:

- *traAFBH* and *traCDG-yci* (Fuqua and Winans, 1996a): controlled by TraR-OOHL bound to *tra box* I, which is located -43.5 nucleotides upstream of the two divergent transcription start sites. These target genes direct DNA processing during conjugation.
- *traI-trb* operon: *traI-trb* encodes genes involved in mating pair formation, the type IV secretion system for DNA transfer and entry-exclusion, as well as the pheromone synthase gene *traI* (Cho *et al.*, 2009; Fuqua and Winans, 1996a). The genes are regulated from *tra box* II which is also between two divergent class II promoters.
- *repABC* operon: The *repABC* genes are responsible for replication and for the partitioning of the Ti plasmid into daughter cells. RepA and RepB are involved in partitioning and RepC is the replication initiator protein (Pinto and Winans, 2011; Zhu *et al.*, 2000). By activating the transcription of the *repABC* operon, the plasmid copy number of the Ti plasmid increases and this enhances the expression of all Ti plasmid-encoded genes (Li and Farrand, 2000; Pappas and Winans, 2003b). TraR controls the expression of three different promoters involved in the regulation of the *repABC* operon (Pappas and Winans, 2003a). *repAP1* is a class II promoter divergent from the *traI-trb* promoter and controlled by *tra box* II (Fuqua and Winans, 1996a; Pappas and Winans, 2003b). The *tra box* II is also used for the activation of a class I promoter, *repAP2,* where the tra box lies -65.5 nucleotides from the transcription start site. The activation of the third

promoter of the *repABC* operon, *repAP3,* is through the binding of TraR to *tra box* III and this is a class II promoter.

- *traM*: the expression of *traM* occurs from *tra box* IV, which is located -65.5 nucleotides from the transcription start site, and is a class I promoter. TraM is an antiactivator protein which inactivates TraR. This protein is the focus of the next section and also of Chapter 4.

The mechanism of transcription activation involves the binding of TraR-OOHL to the *tra* boxes and recruiting RNA polymerase to adjacent promoters. Extensive mutagenesis studies in the N-terminal domain (NTD) and carboxy-terminal domain (CTD) of TraR has revealed surfaces that are important for transcription activation, and which are thought to make direct contact with this enzyme (Costa *et al.*, 2009; Luo and Farrand, 1999; Luo *et al.*, 2003; Qin *et al.*, 2004; Qin *et al.*, 2009; White and Winans, 2005).

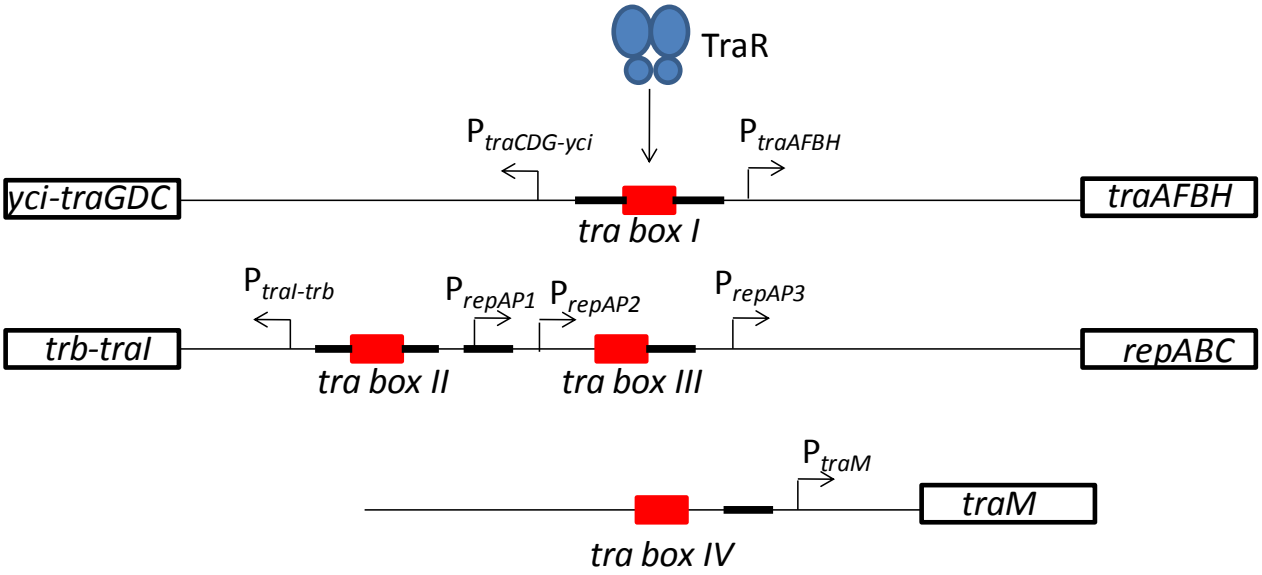

Figure 1.6. Transcription regulation by TraR on the octopine-type Ti plasmid. The TraR regulated genes are shown in the black rectangles and the specific sequences of DNA recognized by TraR are show in red and are indicated as tra boxes I, II, III and IV. The bold bars represent the -10 and -35 regions recognized by the RNA polymerase. The start sites of the transcription are indicated by arrows and the promoter name is indicated at the top of the arrow.

1.5. TraR regulation

The levels of TraR are tightly regulated at transcriptional and post-translational levels. At a transcription level, TraR is the last gene of the operon regulated by OccR. As described above, OccR is a LysR-type transcription factor that detects octopine (Akakura and Winans, 2002; Fuqua and Winans, 1996b; Habeeb *et al.*, 1991; von Lintig *et al.*, 1991). Consequently, OccR is only active in the plant tumor environment, where octopine is abundant. After being transcribed, TraR levels are closely regulated at the post-transcriptional level by the signal molecule OOHL and by the antiactivators TrlR and TraM (Chai *et al.*, 2001; Chen *et al.*, 2007; Zhu and Winans, 1999, 2001).

1.5.1. OOHL

TraR activity requires OOHL, which accumulates only at high bacterial density. OOHL is completely buried in a pocket located in the TraR-NTD (Zhang *et al.*, 2002). The interactions between TraR and OOHL involve both hydrogen bonds and hydrophobic interactions (Chai and Winans, 2004). Structural studies of TraR and experimental studies with whole cells suggest that OOHL is used as a scaffold for TraR folding. Biochemical analysis of the protein showed that when TraR is produced in the bacterial cells in the absence of OOHL (apo-TraR), it is degraded rapidly. Clp and Lon proteases both participate in the degradation of apo-TraR (Zhu and Winans, 2001). Differently from LuxR, which has a reversible binding to its signal molecule

(Urbanowski *et al.*, 2004), binding of TraR to OOHL is irreversible, with a low dissociation constant and a high affinity (Zhu and Winans, 2001). It is believed that TraR folds in a co-translational manner based on the facts that binding to OOHL is irreversible and that the resistance to protease degradation do not change with the addition of exogenous OOHL (Zhu and Winans, 1999, 2001). This is a further indication that OOHL is critical for folding, stability and functionality of TraR.

1.5.2. TraR dimerization

Dimerization also plays a role in the stability of TraR, increasing its resistance to proteolysis (Pinto and Winans, 2009). Both the CTD and NTD domains of TraR are involved in dimerization (Pinto and Winans, 2009; Qin *et al.*, 2000; Zhu and Winans, 2001). The NTD contributes more extensively than the CTD in this process. In the NTD, dimerization occurs between a long alpha-helix (α-9) in one subunit of TraR that forms a parallel coiled coil with the same helix of the opposite subunit (Vannini *et al.*, 2002; Zhang *et al.*, 2002). The amino acid residues necessary for dimerization were mapped using x-ray crystallography and mutations in these amino acids showed increased instability, (Pinto and Winans, 2009; Zhang *et al.*, 2002). The dimerization helices can be viewed in Figure 1.5 and an illustration of TraR proteolysis is shown in Figure 1.7. However, even the dimerized TraR is degraded at a detectable rate (Zhu and Winans, 1999).

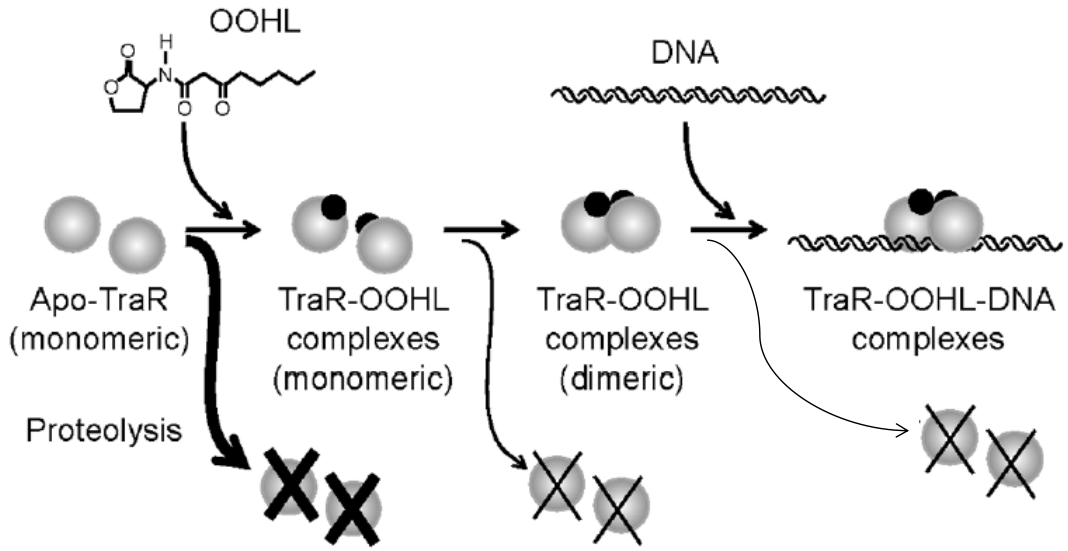

Figure 1.7. Illustration of TraR proteolysis. Apo-TraR is degraded faster than TraR-OOHL. TraR-OOHL monomers are more stable than Apo-TraR, but less stable than TraR-OOHL dimers. Even after dimerization, when TraR is active and can bind to DNA, TraR is degraded at a detectable level. As thicker are the arrows, as faster degradation of TraR occurs. Adapted from Pinto et al., 2009.

1.5.3. Antiactivation of TraR

TraR has two antiactivators, TrlR and TraM, which are encoded by the octopine-type Ti plasmid and inactivate TraR by binding to it (Chai *et al.*, 2001; Chen *et al.*, 2004; Chen *et al.*, 2007; Vannini *et al.*, 2004). This controls the levels of active TraR in the cells.

TrlR

TrlR is part of the mannopine catabolism operon, which is controlled by mannopine, an opine that is composed of mannose conjugated to glutamine, and unrelated to octopine. TrlR is only found in the octopine-type Ti plasmid. Mannopine was found to inhibit conjugation of the Ti plasmid and this inhibition was dependent on the presence of TrlR (Oger *et al.*, 1998; Zhu and Winans, 1998). When TrlR was expressed from a constitutive promoter, it resulted in a decrease of conjugation, indicating that TrlR had a negative effect in the process (Chai *et al.*, 2001).

TrlR is 88% identical to TraR but is truncated and lacks a DNA binding domain, due to a frame shift mutation. Site-directed mutagenesis was used to restore the reading frame of full length gene, resulting in a fully active protein (Zhu and Winans, 1998). TrlR also binds OOHL and it is able to form inactive heterodimers with TraR. These heterodimers do not stably bind DNA (Chai *et al.*, 2001). This effectively inactivates the TraR.

TraM

TraM is an 11 KDa protein that does not have similarity with any other known protein (Hwang *et al.*, 1995; Vannini *et al.*, 2004). The crystal structure of TraM was published in 2004 (Chen *et al.*, 2004; Vannini *et al.*, 2004) and the structure of co-crystals of TraM and TraR was published in 2007 (Figure 1.8) (Chen *et al.*, 2007). TraM binds mainly to helix10 and helix 11 of TraR CTD. The TraM binding forces the two CTDs of TraR apart so they can no longer bind to *tra* box sequences (Chen *et al.*, 2007). In fact, biochemical and genetic studies have identified the amino acids of TraM that interact with TraR and vice-versa confirming the structural model (Qin *et al.*, 2007). It was also shown that a null mutation in TraM causes hyperconjugation and lower concentrations of OOHL are required for the TraR-dependent gene activation. On the other hand, the overexpression of TraM causes the opposite effect (Fuqua *et al.*, 1995; Hwang *et al.*, 1995). Other studies show that the anti-activation effects of TraM occur through the contact between the antiactivator and TraR-OOHL (Hwang *et al.*, 1999; Swiderska *et al.*, 2001) An interesting observation is that in both nopaline and octopine type Ti plasmids, TraR-OOHL activates the transcription of *traM*, providing a negative feedback loop (Fuqua *et al.*, 1995; Hwang *et al.*, 1995). Interestingly, TraR is not the only protein that seems to regulate TraM. In fact, phospho-VirG was found to induce the expression of TraM (Cho and Winans, 2005). As discussed before, phospho-VirG activates the expression of the *vir* operon which is responsible for the processing and transfering of the T-DNA to the plant host. The

expression of TraM by phospho-virG is perhaps important to avoid simultaneous expression of the *vir* and *tra* system because both produce type IV secretion systems to transfer DNA and they might interfere with each other. This also makes sure that plant transformation occurs before bacterial conjugation (Cho and Winans, 2005). The fate of the proteins after the antiactivator binds to TraR is one of the topics examined in detail in this dissertation (Chapter 4).

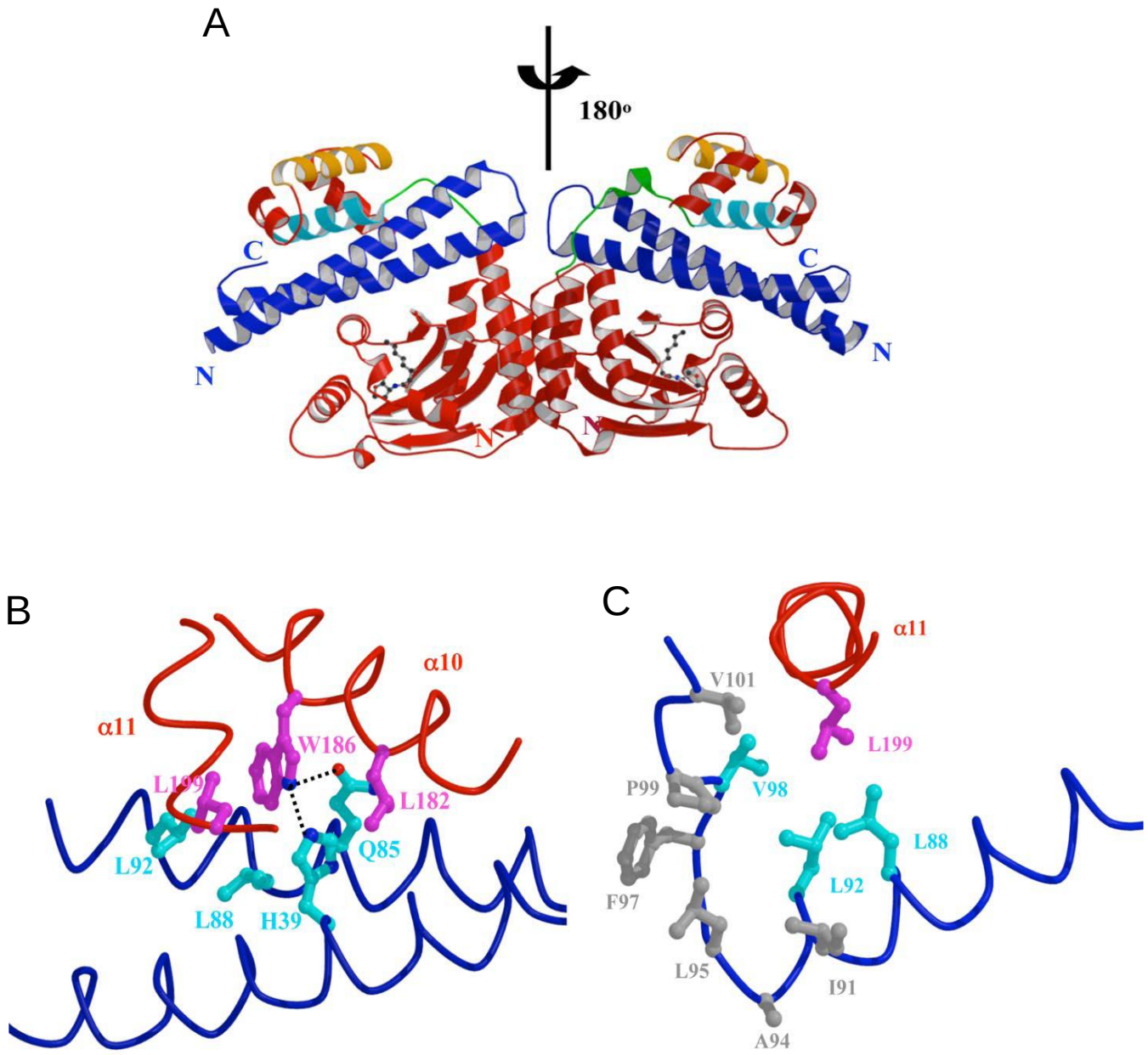

Figure 1.8. Interactions between TraM and TraR. A) Model of co-crystal between dimer TraM, shown in blue and dimer TraR, shown in red. B) TraM side chains are shown in cyan and the helices in blue. TraR helices that contact TraM are shown in red and the side chains in magenta. The interactions between residue W186 of TraR and TraM are shown where the oxygen atom can be seen in red and the nitrogen atoms in blue. C) The interactions between residue L199 of TraR and a hydrophobic cluster at the CTD of TraM. The hydrophobic side chains are shown in gray and the residues that directly contact L199 in cyan (Chen *et al.*, 2007).

1.6. Contents of the dissertation

TraR is the predominant focus of this dissertation. In Chapter 2, site-directed mutagenesis studies are carried out in order to identify the amino acids in the NTD of TraR that are critical for the interaction between TraR and the RNA polymerase. These studies allow the identification of patches of amino acids on the surface of TraR that mediate the interaction. This allows a better understanding of how transcription activation occurs at a molecular level.

Chapter 3 deals with the susceptibility of TraR to proteolysis. TraR abundance is subject to a number of factors, including transcription of the *traR* gene in response to octopine, and by proteolytic degradation. Apo-TraR is rapidly destroyed, and TraR-OOHL complexes that fail to dimerize are also degraded. Even dimeric TraR is still subject to proteolysis. In Chapter 3, I examine the proteolysis of TraR in detail: in particular, I investigate which regions of TraR provide targets for cellular proteases. Two recognition motifs in the CTD of TraR were found. Mutation studies revealed that the protein was more stable when these regions were mutated or when the regions were removed.

In Chapter 4 I consider the antiactivator protein of TraR, TraM. As discussed above (Section 1.5), it is known that TraM inactivates TraR by forming a 2:2 complex. The particular focus of my study is to examine the fate of these complexes. The results indicate that TraR in these complexes is

targeted for proteolysis, while TraM in the same complexes is spared from proteolysis, and can act catalytically to cause degradation of additional TraR dimers.

Chapter 5 contains the conclusions and a discussion of future directions.

Appendix 1 considers a different transcription activator from the LuxR family, CepR. This transcription activator is found in *Burkholderia cenocepacia,* an opportunistic human pathogen. My contribution to this collaborative work was to mutagenize a CepR binding site, in order to identify bases that are required for CepR binding. Others in this project used this information to define a consensus CepR binding site and to identify new CepR-regulated promoters.

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[1]CHAPTER 2

Identification of Amino Acid Residues of the Pheromone-binding Domain of the Transcription Factor TraR that are Required for Positive Control

2.1. Summary

Genes required for replication and for conjugal transfer of the *Agrobacterium tumefaciens* Ti plasmid are regulated by the quorum sensing transcription factor TraR, whose N-terminal domain binds to the pheromone *N*-3-oxooctanoyl-L-homoserine lactone (OOHL) and whose C-terminal domain binds to specific DNA sequences called *tra* boxes. Here, we constructed 117 mutants, altering 103 surface-exposed amino acid residues of the TraR N-terminal domain. Each mutant was tested for activation of the *traI* promoter, where TraR binds to a site centered 45 nucleotides upstream of the transcription start site, and of the *traM* promoter, where TraR binds a site centered 66 nucleotides upstream. Alteration of 18 residues blocked activity at the *traI* promoter. Of these, alteration at three positions impaired TraR abundance or DNA binding, leaving 15 residues that are specifically needed for positive control. Of these 15 residues, nine also blocked or reduced activity at the *traM* promoter, while six had no effect. Amino acid residues required for

[1] Costa, E.D., Cho, H., and Winans, S.C. (2009) Identification of amino acid residues of the pheromone-binding domain of the transcription factor TraR that are required for positive control. *Mol Microbiol* **73(3): 341-351.** Table 2.4 and Fig. 2.4 were done by Cho, H. All the other figures and Tables were done by Costa, E.D.

activation of both promoters probably contact the carboxy terminal domain of the RNA polymerase α subunit, while residues required only for *traI* promoter activation may contact another RNA polymerase component.

2.2. Introduction

Many species of bacteria use diffusible pheromones to coordinate a wide range of physiology, including pathogenesis, sporulation, the formation of biofilms, and horizontal transfer of DNA (Whitehead *et al.,* 2001; Winans and Bassler, 2002). One such regulatory system is composed of the LuxI and LuxR proteins of *Vibrio fischeri*, where LuxI synthesizes 3-oxo-hexanoylhomoserine lactone (OHHL) (Eberhard *et al.,* 1981), while LuxR is an OHHL receptor and OHHL-dependent transcription factor of the organism's bioluminescence operon. In the past 15 years, a wide variety of related systems have been discovered, and a small number of them have been intensively studied. In most cases, a LuxI homolog is functionally paired with a LuxR homolog, in that the former synthesizes an acylhomoserine lactone (AHL) while the latter is a transcription factor that binds the cognate AHL. In most cases, DNA binding activity requires the AHL (Pappas *et al.*, 2004), while in a few cases, the AHL has the opposite effect, blocking DNA binding (Castang *et al.,* 2006; Cui *et al.,* 2006; Fineran *et al.,* 2005; Sjoblom *et al.,* 2006). Biochemical, genetic, and structural studies of a number of LuxR homologues have revealed that they are two-domain proteins, whose N-

terminal domain (NTD) binds AHLs and whose C-terminal domain (CTD) binds DNA (Pappas *et al.,*2004). Both domains contribute to protein dimerization (Luo *et al.,* 2003; Vannini *et al.,* 2002; Zhang *et al.,* 2002).

The Ti plasmid of the plant pathogen *Agrobacterium tumefaciens* encodes such a regulatory system, consisting of the AHL synthase TraI and the transcription factor TraR. TraI synthesizes primarily 3-oxo-octanoylhomoserine lactone (OOHL) while TraR is an OOHL-dependent activator of three closely spaced promoters of the *repABC* operon, which directs plasmid replication and partitioning, and of three promoters of the *tra* and *trb* operons, which are required for conjugative transfer of the Ti plasmid (Piper *et al*., 1993; Zhang *et al.,* 1993; Fuqua and Winans, 1994; More *et al.,* 1996; Li and Farrand, 2000; Pappas and Winans, 2003). TraR-OOHL complexes bind to 18-nucleotide dyad-symmetrical sequences called *tra* boxes to activate transcription of target promoters (Winans *et al.*, 1999).

Genetic, biochemical and structural studies of TraR and its interactions with OOHL and DNA have made this protein an intensively studied representative of the LuxR family. TraR monomers bind to OOHL in a 1:1 mole ratio and form homodimers that bind to *tra* boxes with high affinity and specificity (Luo and Farrand, 1999; Zhu and Winans, 1999). The N-terminal pheromone-binding domain of TraR is sufficient for OOHL binding and dimerization, as TraR fragments containing just this domain are able to form inactive heterodimers with full-length protein (Chai *et al.,* 2001; Luo *et al.,* 2003; Qin *et al.,* 2000; Zhu and Winans, 1998). Binding of OOHL to TraR is

virtually irreversible, as this pheromone is buried deeply within the protein and makes no contact with bulk solvent. Activity of TraR, LuxR, and a few other members of this family have been reconstituted *in vitro*, and require only promoter DNA, the activator complexed with its AHL, and RNA polymerase (RNAP) (urbanowski *et al.,* 2004; Zhu and Winans, 1999).

The quaternary structure of TraR, OOHL, and *tra* box DNA has been solved by X-ray crystallography (Vannini *et al.*, 2002; Zhang *et al.*, 2002). This structure confirmed the domain structure of this dimeric protein. Protein dimerization is achieved largely by a rather long alpha helix on each of the two the N terminal domains, which together create a coiled coil, and by two shorter helices in the C terminal domains (CTD), which create a second coiled coil. The CTD is a four-helix bundle with a helix-turn-helix DNA binding motif (HTH) common to many prokaryotic regulators (Nelson, 1995). This four-helix domain structure is common to all members of the LuxR-NarL-FixJ superfamily of prokaryotic transcriptional regulators (Fuqua and Greenberg, 2002). Several amino acid residues found on the recognition helix make specific contact with *tra* box DNA sequences
(Vannini *et al.*, 2002; White and Winans, 2007; Zhang *et al.*, 2002).

There are seven known TraR-dependent promoters on the octopine-type Ti plasmid, all of which contain *tra* boxes. At five of these promoters (P*traA,* P*traC,* P*traI,* P*repA1, and* P*repA3)* the TraR-binding site is centered approximately 45 nucleotides upstream of the transcription start site, adjacent to the -35 elements of these promoters. At the remaining two

promoters (P*repA2* and P*traM*), the *tra* box is located approximately 66 nucleotides upstream.

The first class of promoters are reminiscent of class II-type promoters first described for CAP (Busby and Ebright, 1999) while the second class of promoters resemble class I-type promoters. Activators of class II-type promoters can interact with several different subunits of RNAP, while activators of class I-type promoters are generally thought to interact with the C-terminal domain of the alpha subunit of RNAP (α-CTD), which is connected to α-NTD by a flexible linker (Busby and Ebright, 1999). A number of other activators have been shown to contact either the α or σ subunits via their DNA binding domains (Busby and Ebright, 1999; Bushman *et al.,* 1989; Crater and Moran, 2002; Danot *et al.,* 1996; Stibitz, 1994).

In a previous study (White and Winans, 2005), we used TraR structural data to alter all surface exposed amino acids of the C-terminal domain, and tested each for defects in activating a class I promoter and a class II promoter (White and Winans, 2005). Alteration of six amino acid residues abolished activation of both promoters, without significantly affecting protein accumulation or DNA binding. As these mutants were defective at both classes of promoter, we concluded that the residues of the wild type TraR most likely interacted with the α CTD.

Two residues in the N-terminal domain of the pTiC58 TraR protein are critical for activation of a class II type promoter (Luo and Farrand, 1999).

Mutations of these residues (D10 and G123) disrupted contacts with purified α-CTD (Qin *et al.*, 2004). It seemed plausible that other residues in the N-terminal domain of TraR might also make specific contacts with RNAP. In order to study the role of the TraR N-terminal domain in transcription activation, we performed saturating site-directed mutagenesis of all surface-exposed TraR residues. Each mutant was tested for *in vivo* activity at a class II promoter, for its accumulation *in vivo*, and for ability to bind DNA *in vitro*. We identified 15 mutations that accumulate and bind to DNA but that fail to activate the class II promoter. Of these, 9 mutants also abolish activation of the class I promoter, while the remaining 6 mutants affected only the class II promoter. From these data, and from the positions of these mutations on the surface of TraR, we speculate that the residues needed for both class I and class II promoters are likely to interact with α CTD of RNAP, while the residues needed only for the class II promoter are likely to interact with another RNAP subunit.

2.3. Results

Mutagenesis of residues in the N-terminal domain of TraR

As described above, we sought to determine whether the N-terminal domain of TraR contains regions likely to make direct contact with RNA polymerase. We used the X-ray crystal structure of TraR (Vannini *et al.*, 2002; Zhang *et al.*, 2002) to identify all solvent-exposed residues on this part of the protein, and used site-directed mutagenesis to alter each residue (Vannini *et al.*, 2002; Zhang *et al.*, 2002). Seven residues of the linker domain were also included in this study. We have previously found that many alterations of surface residues of TraR can cause a significant decrease in protein accumulation, indicating that the mutants were sensitive to proteolysis (White and Winans, 2005). In an effort to minimize this problem, we made substitutions wherever possible that preserved interactions with adjacent amino acid residues, but that altered the surface exposed to solvent. In all, 117 point mutants were constructed at 103 positions, saturating the surface of the TraR NTD. These mutants are listed in Table S1 and also indicated in Fig. 2.1.

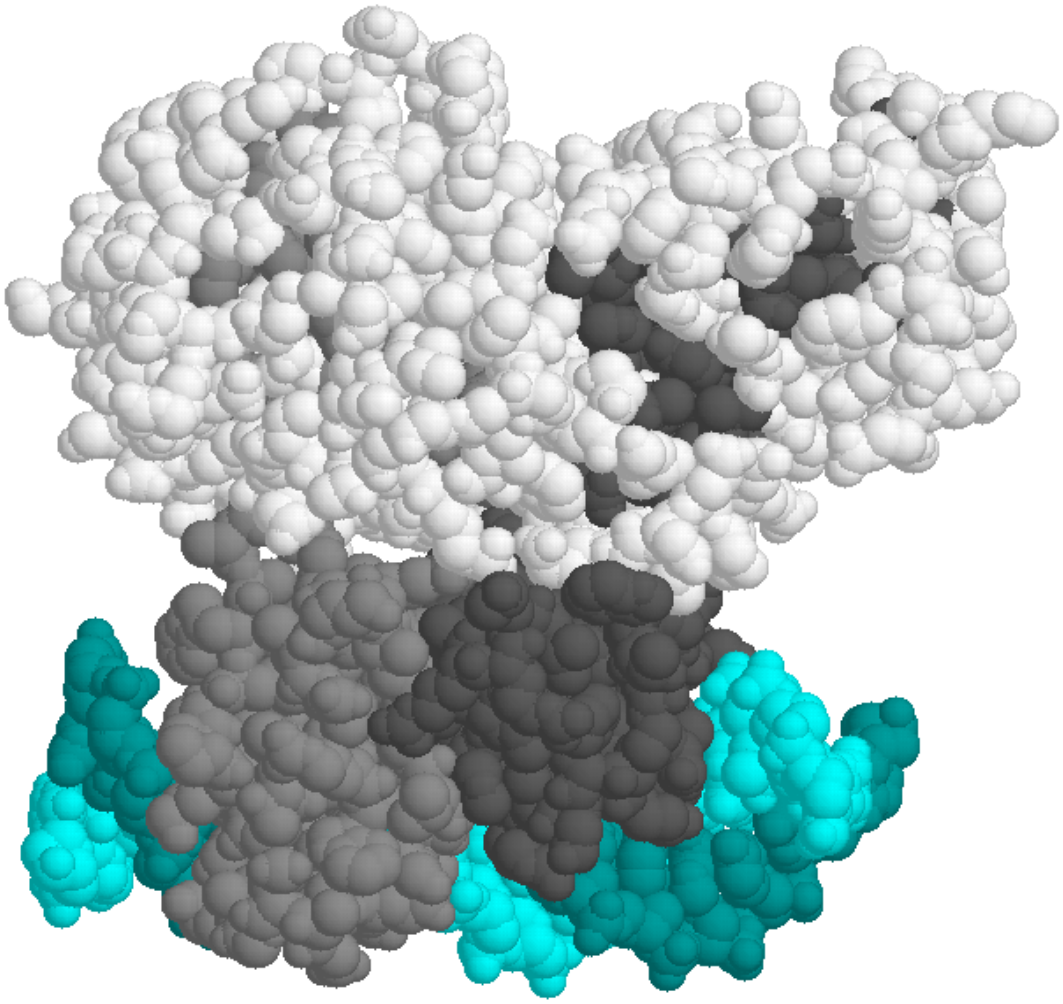

Figure 2.1. Saturation mutagenesis of the surface of the NTD of TraR. Residues in white were altered by site-directed mutagenesis.

Activity of TraR mutants *in vivo*

Each TraR mutant was tested for its ability to activate transcription of a TraR-dependent promoter. We used *A. tumefaciens* strain NTL4 (pCEW260), which lacks a Ti plasmid and thus does not have the native *traR* or *traI* genes (Luo *et al.*, 2001). Plasmid pCW260 contains a P*traI-lacZ* fusion, and has a wild type *traI* promoter except for a one-nucleotide mutation in its *tra* box. This mutation creates a consensus *tra* box with perfect dyad symmetry (White and Winans, 2005). The wild type TraR protein expressed this fusion at high levels (approximately 3000 Miller units) in the presence of saturating levels of OOHL (100 nM), while its activity in the absence of either TraR or OOHL was barely detectable (less than 5 Miller units). OOHL was added at four different concentrations (0.1 nM, 1 nM, 10 nM and 100 nM) to each culture and assayed for β-galactosidase activity 8 hours later. The resulting expression levels for all 117 mutants are shown in Table S1. Twenty mutants, together altering 18 residues, showed less than 50% of wild type activity in the presence of 100 nM OOHL (Table 2.1).

Table 2.1. Mutations in the TraR N Terminal Domain that are defective in transcription[a]

Mutation	*traI* Promoter Activity at the Indicated OOHL Concentrations			
	0.1 nM	1 nM	10 nM	100 nM
Wild type	(100)[b]	(100)[b]	(100)[b]	(100)[b]
Vector Control	<1	<1	<1	<1
D6E	20	15	28	42
D6G	<1	<1	<1	<1
K7R	9	31	28	44
D10N	<1	<1	<1	<1
A13L	<1	1	20	7
E15K	6	18	31	46
D17E	12	11	34	21
I20W	5	12	38	14
H44K	5	18	34	42
P71H	<1	<1	<1	8
K74E	<1	6	16	8
R75E	8	32	41	48
R77E	<1	<1	2	1
S78E	12	26	22	28
R79E	32	48	36	36
K80E	3	19	24	23
N122A	<1	3	22	29
N122D	<1	1	10	16
G123R	<1	<1	<1	<1
D144R	<1	<1	5	23

a: All mutations included in this list functioned at levels 50% or lower than wild type TraR in the presence of 100 nM OOHL. Data are presented as a percentage of wild type.

b: Wild type TraR expressed the promoter, a *traI-lacZ* fusion, at 700, 1500, 2600 and 3000 units of β-galactosidase activity at 0.1 nM, 1 nM, 10 nM and 100 nM of OOHL, respectively.

In vivo accumulation of mutant proteins

In previous studies, we found that some TraR point mutants defective in activation also failed to accumulate *in vivo* (White and Winans, 2005). We therefore used semi-quantitative Western immunoblotting to test all mutants for their ability to accumulate *in vivo*. These assays were done using the same *A. tumefaciens* strain as used for activity assays described above. Equivalent amounts of crude cell extracts were loaded into each lane. A representative Western is shown in Fig. 2.2, and the data expressed in per cent accumulation compared with wild type for all mutants are summarized in Table S1. Results for the 20 mutants described above are shown in Table 2.2.

Of the 20 mutants described in Table 2.1, three mutants accumulated at levels less than 45% of wild type (Table 2.2). Conversely, 17 mutations, altering 16 different residues, did not cause a decrease in abundance commensurate with their decrease in activity. These 16 mutations are expected to cause defects in some other aspect of transcription activation, possibly either DNA binding or interactions with RNAP.

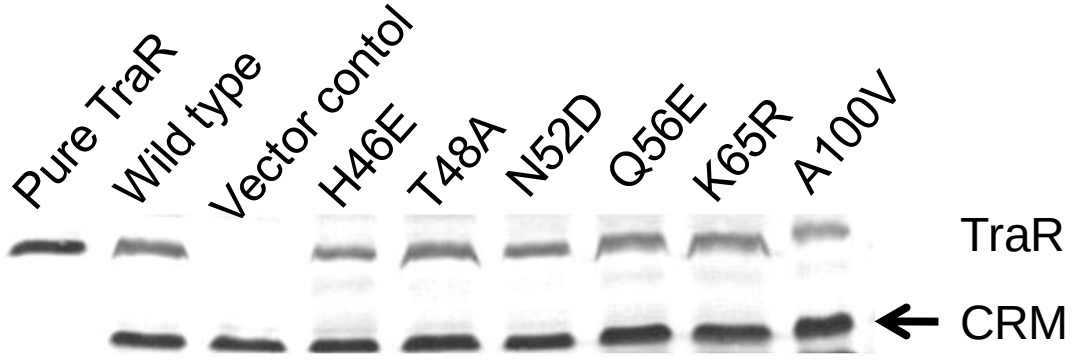

Figure 2.2. Western immunoblot data of TraR point mutants in *A. tumefaciens* strain NTL4 (pCEW260). Strains containing pYC335 or pPZP201served as positive and negative controls, respectively. Purified TraR was also used as a positive control for all westerns (left lane). The cross-reacting material (CRM) was used to normalize the intensity of each TraR band.

Table 2.2. Accumulation in *A. tumefaciens* and DNA-binding affinity *in vitro* of all transcription-defective mutants.

Mutation	Accumulation (percent of wild type)	DNA binding (percent of wild type)
Wild type	(100)	(100)
Vector Control	<1	<1
D6E	120	89
D6G	102	70
K7R	78	80
D10N	101	109
A13L	100	65
E15K	45	74
D17E	70	30
I20W	49	57
H44K	27	20
P71H	<10	NT[a]
K74E	76	74
R75E	88	41
R77E	77	72
S78E	100	103
R79E	68	63
K80E	92	65
N122A	61	100
N122D	33	35
G123R	72	110
D144R	82	74

a: NT, not determined.

Ability of TraR mutants to bind DNA fragments containing *tra* box sequences

We conducted electrophoretic mobility shift assays (EMSA) using a radiolabeled DNA fragment and cleared cell lysates containing TraR point mutants. The strains were cultured in the presence of OOHL and Western blots were performed with each cleared cell lysate (data not shown). The volumes of each lysate added to the gel were adjusted using the Western immunoblot data described above. The DNA fragment used in these experiments contained the *traA-traC* intergenic region, which contains a consensus binding site for TraR. Mutants that had severe defects in accumulation in *A. tumefaciens* (see above) also did not accumulate well in the protease-deficient strain KY2347, and were therefore not included in the gel mobility shift assays. Representative EMSA data are shown in Fig. 2.3, while the data for all mutants are summarized in Table S1. Complexes were not detected using an extract lacking TraR (Fig. 2.3, lanes labeled vector control). Of the 20 transcription-defective mutants described in Table 2.1, three bound the *tra* box DNA fragment at levels less than 40% of wild type (Table 2.2). For these mutants, D17E, H44K, and N122D, the defect in DNA binding could explain the defect in transcription. However, two of these mutants were also defective in accumulation. In all, a total of 16 mutants, altering 15 residues, showed defects in transcription that could not be accounted for by a lack of accumulation or an inability to bind *tra* box DNA.

These mutants are therefore candidates for having defects in interactions with RNA polymerase.

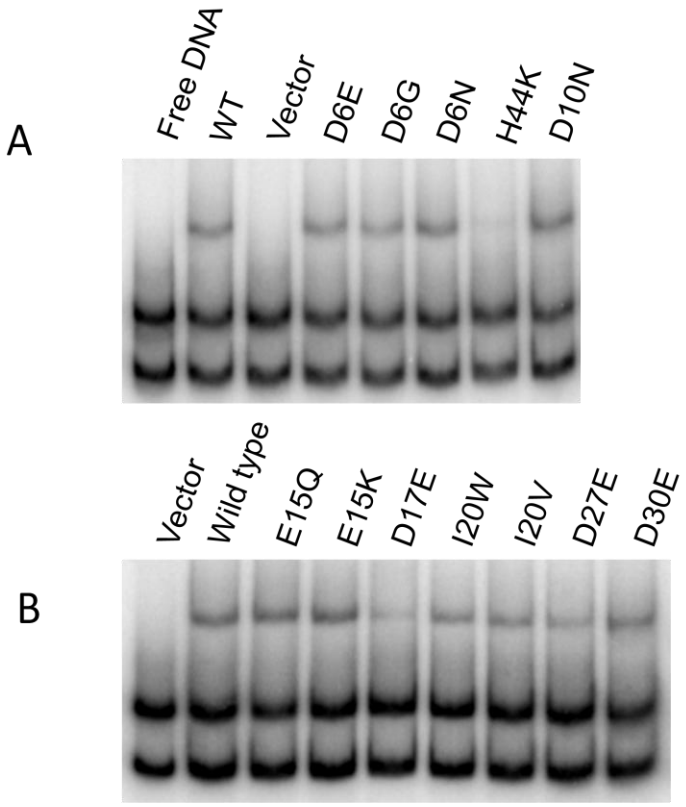

Figure 2.3. Electrophoretic mobility shift assays with TraR in crude cell extracts. The amount of full-length, soluble TraR in each extract was normalized using western immunoblots.

Table 2.3. Activity of TraR mutants at the TraM promoter.

Mutation	Activity at P*traI* 100 nM	Activity at P*traM* 100 nM	TraR-dependent P*traM* activity
Wild Type	(100)	(100)	90
Vector	<1	11	0
D6E	42	78	67
D6G	<1	16	5
K7R	44	75	64
D10N	<1	11	0
A13L	7	104	93
E15K	46	98	87
D17E	21	70	59
I20W	14	100	89
H44K	42	27	16
K74E	8	36	25
R75E	48	40	29
R77E	1	15	4
S78E	28	37	26
R79E	36	46	35
K80E	23	102	91
N122D	16	33	22
N122A	29	62	51
G123R	<1	35	24
D144R	23	47	36

Activity of site-directed mutants at a class I promoter

The activity assays described above were all done with a class II promoter, where TraR could interact with several different subunits of RNAP. We also tested these mutants at the *traM* promoter, which is a class I promoter. TraR activating such a promoter should in principle interact only with α CTD. The promoter we used was identical to the wild type P*traM* promoter except that its *tra* box contained the consensus TraR binding sequence (White and Winans, 2005).

This promoter has a TraR-independent basal expression of approximately 120 Miller units, and is activated only approximately seven-fold by TraR in the presence of saturating OOHL concentrations. Since this induction ratio is rather low, we tested the mutants using saturating levels of OOHL (100 nM) and deducted the TraR-independent activity of this promoter. Many of the TraR mutants that were impaired at *traI* were also impaired at P*traM* (Table 2.3). The clearest examples of this include D6G, D10N, K74E, R77E, and G123R, while other possible examples include R75E, S78E, R79E, and D144R. In a previous study, additional positive control mutations were isolated in the TraR CTD, all of which blocked activity at both types of promoters (White and Winans, 2005).

A few of the mutants that were defective at the *traI* promoter were unimpaired at the P*traM* promoter (Table 2.3). The clearest examples of this are A13L, I20W, and K80E, while other possible examples include K7R, E15K,

and N122A. These phenotypes suggest that the wild type residues at these positions contact RNAP at the *traI* promoter but make no critical contacts with RNAP at the *traM* promoter.

Intragenic complementation of TraR mutants

Dimeric transcription factors ought in principle to have two activating regions (ARs) per dimer. If an AR is composed of amino acids from both subunits, it may be possible to restore protein function by co-expressing two defective proteins (see Fig. 2.4). The data described above, taken in conjunction with structural information, suggested that the AR that contacts α-CTD might be composed of amino acid residues of both TraR subunits. To test this, we co-expressed various TraR PC mutants and assayed for TraI promoter activity. One of these mutations, W184H, lies in the TraR-CTD and has a strong positive control defect (White and Winans, 2005). When mutant D10N was co-expressed with W184H, no complementation was observed (Table 2.4). This suggests that one mutation disrupted one of the two ARs in the TraR dimer, while the other mutation disrupted the second AR (Fig. 2.4). W184 and D10 of each AR must therefore be contributed by the same subunit. In contrast, when mutants G123R and W184H were co-expressed, activity was almost fully restored (Table 2.4). This suggests that W184 and G123 of each AR are contributed by different subunits, and that in the merodiploid strain, each heterodimer has one functional site and one doubly non-functional one (Fig. 2.4).

Table 2.4. Intragenic complementation of positive control mutations of TraR.

pPZP201 derivative	pBBR1MCS5 derivative	β-galactosidase Activity
wild type	wild type	2343
vector	vector	2
D10N	G123R	425
G123R	D10N	334
D10N	W184H	45
W184H	D10N	34
G123R	W184H	1556
W184H	G123R	1408

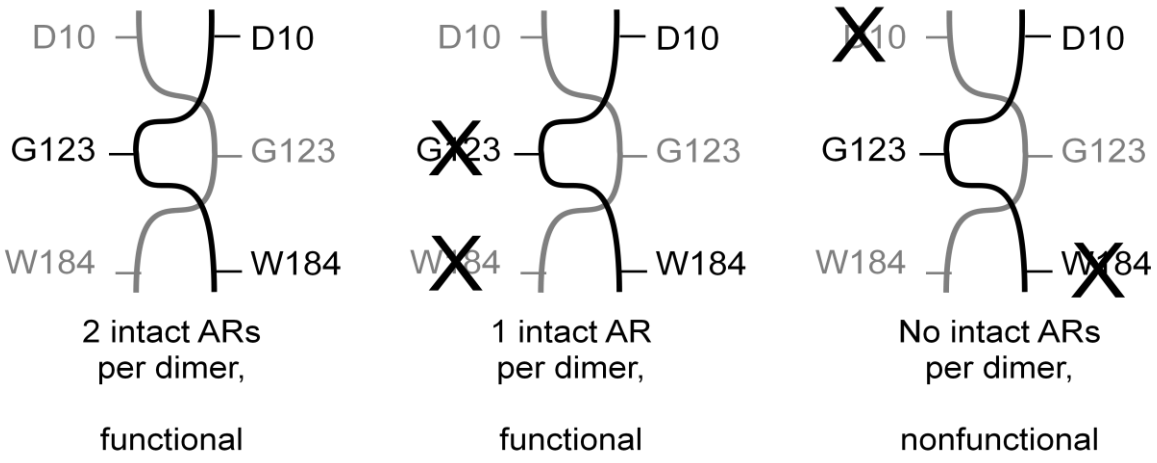

Figure 2.4. Intragenic complementation of mutations of D10, G123, and W184. Successful complementation was taken as an indication that the amino acid residues are contributed by opposite subunits.

In a third experiment, mutants D10N and G123R were co-expressed. Activity was restored, but not to wild type levels (Table 2.3). The restoration of activity suggests that D10 and G123 of each AR are contributed by different

subunits. The fact that restoration was incomplete suggests that the two mutations on one AR somehow weakened the activity of the opposite AR, possibly via a perturbation of the quaternary structure of the TraR dimer.

2.4. Discussion

In a previous study, we saturated the surface of the TraR CTD with point mutations, and identified a contiguous patch of residues that are essential for activation of Class I and Class II promoters (White and Winans, 2005). In the present study, we sought to identify one or more similar patches on the TraR NTD. Two positive control mutations in the TraR NTD have previously been described by another group (Qin *et al.*, 2004). Saturation mutagenesis of the surface of the TraR NTD led to the discovery of a significant number of residues that are required for positive control. Each such residue may make direct contacts with RNAP.

TraR is probably a highly flexible molecule, with two NTDs tethered to two CTDs by a flexible linker. If so, then the spatial position of the two NTDs of the dimer with respect to the two CTDs could be highly variable at different promoters. We believe that the CTDs of the TraR dimer, once bound to *tra* box DNA, can then recruit RNAP to the promoter. Different surfaces of the TraR NTDs could then “explore” the surface of any proximal RNAP surface until a patch of TraR binds a patch of RNAP.

Assuming that residues required for positive control do contact RNAP, we would like to begin to map these interactions, identifying which subunit of RNAP is contacted by each residue. Presumably, residues required for activation of both class I and class II promoters most likely contact the α-CTD, while residues required only for class II promoters are more likely to contact some other RNAP subunit. We have divided all positive control mutants into two groups: (i) those that affect the Class I and Class II promoters, and (ii) those that affect only the Class II promoter. Residues of the former class are likely to contact α-CTD and are shown in white in Figures 2.5 and 2.6, while the latter class are likely to contact another RNAP subunit and are shown in black.

The asymmetry of crystallized TraR creates what we will refer to as a “concave” surface and a “convex” surface (Fig. 2.5). In many cases, residues that are quite closely packed on the concave surface are widely dispersed on the convex surface. For example, W184 of one subunit and G123 of the other subunit lie less than 5 angstroms apart on the concave surface, but lie 55 angstroms apart on the convex surface. All the amino acid residues that are thought to contact α-CTD are closely spaced on the concave surface, while they are widely separated on the convex surface. As a working model, we propose that the asymmetry of cyrstalized TraR resembles to some degree the active form *in vivo*, and that the concave surface of TraR resembles the surface that contacts α-CTD. If so, all four domains of the TraR dimer contribute residues to this putative patch. Three are contributed by the NTD of

the A subunit (D6, D10 and D144), six are contributed by the NTD of the B subunit (K74, R75, R77, S78, R79, and G123), five are contributed by the CTD of the A subunit (W184, V187, K189, E193 and V197), and one is contributed by the CTD of the B subunit (D217). Some of these residues are somewhat sheltered from solvent, especially D6, D10, but would be far more exposed to solvent if the NTD and CTD were to separate slightly. Intragenic complementation analysis supports this model, as it demonstrated that residues G123 and D10 are contributed by opposite subunits, as are residues G123 and W184, while W184 and D10 are contributed by the same subunit.

Of the fifteen residues of this patch, five are basic and five are acidic. As described above, these residues most likely interact with α-CTD of RNAP. Given the flexibility of the TraR linker, the NTD and CTD of this protein should be able to separate, so that the residues contributed by the two CTDs could bind one face of α-CTD of RNAP, while the residues contributed by the two NTDs could bind another face. The α-CTD of RNAP of *A. tumefaciens* has a pronounced acidic patch that could conceivably bind the basic residues K74, R75, R77, and R79 (data not shown).

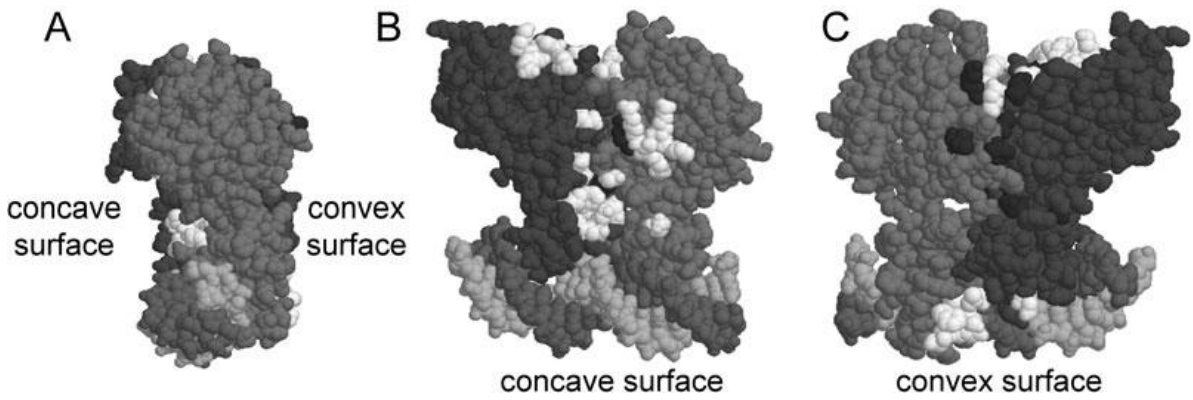

Figure 2.5. Positive control mutants isolated in this study (in the TraR NTD) and in a previous study (TraR-CTD) (White and Winans, 2005). Residues in white affect both P*traI* and P*traM*, while residues in black affected only P*traI*. (A) View of TraR along the DNA axis, showing the concave and convex faces of the crystal structure. (B and C) TraR rotated to show the concave surface and convex surfaces, respectively.

Four residues (W184, V187, K189, and V197) are located at or near the turn between α-helix 10 and α-helix 11 (the scaffold helix of the helix-turn-helix motif). Very similar activating regions have been identified in a number of other regulators with HTH motifs, including CAP, FNR, SoxS, and 8C1 (Bell and Busby, 1994; Busby and Ebright, 1999; Bushman *et al.,* 1989; Griffith and Wolf, 2002). In all of these cases, the activating region includes a surface-exposed loop similar to that of TraR. Similar ARs have been identified in the DNA-binding domains of the NarL homologues GerE and BvgA (Crater and Moran, 2002; Stibitz, 1994). The activating region of FNR that contacts α-CTD is also extensive and spans across three surface-exposed loops, while the FNR homologue CAP contacts the α-CTD with just one loop (Bell and Busby, 1994; Busby and Ebright, 1999; Li *et al.,* 1998; Williams *et al.,* 1997). .

Although we predict that the two activating regions described above contact the α-CTD, the positions of these patches are intriguing. In the CAP-αCTD-DNA ternary structure, the α-CTD binds in the minor groove directly downstream of CAP in class I promoters, and directly upstream of CAP in class II promoters. Contacts are therefore made using the "downstream surface" or "upstream surface" of CAP, respectively. In contrast, the activating regions of TraR lie neither on the upstream nor downstream surfaces of the protein, but rather on a side surface, suggesting that α-CTD could bind DNA to one side of TraR (Fig. 2.5B). This was reported to be the case for the BvgA protein of *Bordetella pertussis* (Boucher *et al.*, 2003).

Some of the positive control mutants isolated in this study affected only the Class II promoter (indicated in black in Figures 2.5 and 2.6). The simplest interpretation is that all or at least most of these mutations block interactions between TraR and a portion of RNAP other than α-CTD. One problem with this interpretation is that some of these mutants overlap regions that were interpreted as binding α-CTD. For example, the mutation K80E blocked P*traI* expression, but had no effect on P*traM* (Table 2.3). However, residue K80 is adjacent to residues K74-R79, which were needed for both promoters. Similarly, residues K7, A13, E15, I20 of one subunit, and N122 from the other subunit, are needed only for P*traI*, yet they roughly encircle residues D10 and D6, which are needed for both promoters. Nevertheless, it is tempting to speculate that K7, A13, E15, I20, and N122 form a contiguous patch on the convex surface of the TraR dimer, and that this patch contacts RNAP. If so,

alteration of D6 or of D10 might conceivably cause a more general loss of function of the protein, blocking activity at both promoters.

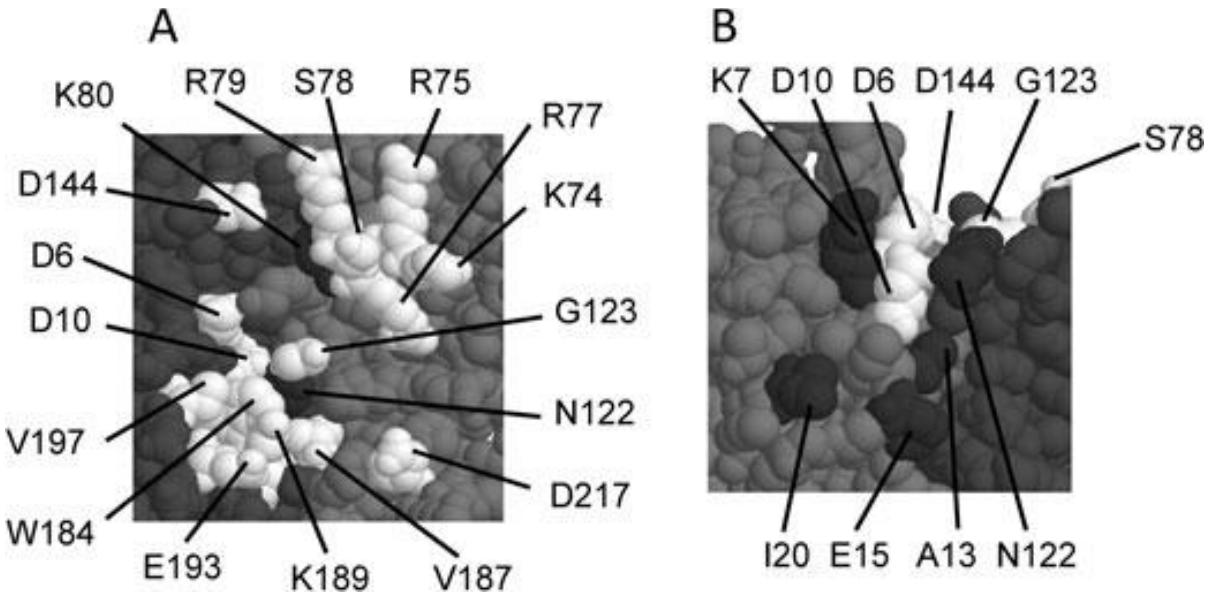

Figure 2.6. Closeup views of TraR residues required for positive control. Residues in white are needed for both promoters, while residues in black are needed only for P*traI*. (A) A patch on the concave surface, composed of residues from both subunits and both domains of each subunit. (B) A patch on the convex surface, composed of residues from the NTDs of both subunits.

The mutations that affect both promoters may be somewhat analogous to the AR1 region of the CAP protein of *E. coli* (Busby and Ebright, 1999; Lawson *et al.,* 2004; Niu *et al.,* 1996). AR1 (residues 156-164 and R209) of CAP interacts with the “287 determinant” of α (residues 283-288 and 314-317, all lying within the αCTD). In contrast, TraR residues that contact α-CTD are thought to lie on both the TraR CTD and the TraR NTD. AR2 of CAP (residues 19, 21, 96, and 101, which form a contiguous patch) interacts with residues

162-165 of α (within the αNTD). AR3 of CAP (residues 52-58) interacts with residues 593-603 of α (lying within region 4, which also decodes the -35 promoter element). At Class I promoters, only AR1 contacts RNAP, while at Class II promoters, AR1, AR2 and AR3 can all make productive contacts. AR1 of CAP acts by recruiting RNA polymerase to promoters, while AR2 and AR3 are thought to function by a combination of recruitment and postrecruitment mechanisms such as promoter melting.

The LuxR CTD has been extensively mutagenized in a search of positive control mutants
(Egland and Greenberg, 2001; Trott and Stevens, 2001). Three residues essential for activation but not for DNA binding were described, K198, W201, and I206, which align with residues K189, E192, and V197 of TraR, respectively. K189, E193, and V197 of TraR are essential for positive control, indicating that these positive control regions overlap. The LuxR-NTD has not so far been studied at this level. The fact that LuxR functions in *E. coli* has been exploited by using libraries of alanine scanning mutants of the RNAP α and σ subunits. Mutations of σ residues 591, 595, 597, 602, and 603 strongly inhibited LuxR-dependent gene expression (Johnson *et al.*, 2003). Many of these residues were also critical in interactions between σ and AR3 of CAP (Lawson *et al.,* 2004). In a separate study, alteration of α-CTD residues 262, 265, 290, 295, 296 and 314 inhibited LuxR activity. These residues overlap the 287 determinant, which interacts with AR1 of CAP (Lawson *et al.*, 2004). These studies using screening of alanine-scanning mutants of LuxR or RNAP

subunits thus provided evidence that LuxR binds both the α-CTD and σ subunits of the RNAP (Finney *et al.,* 2002; Johnson *et al.,* 2003; Stevens *et al.,* 1999).

2.5. Experimental Procedures

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 2.5. *A. tumefaciens* strains were cultured in AT minimal medium at 28°C (Tempe *et al.*, 1977). *Escherichia coli* strains were cultured in LB medium. Synthetic OOHL was provided by A. Eberhard (Cornell University). Antibiotics were added to maintain plasmids at the following concentrations: 100 µg/mL spectinomycin, and 50 µg/mL kanamycin for *E. coli*; and 200 µg/mL spectinomycin and 200 µg/mL kanamycin for *A. tumefaciens*.

Table 2.5. Strains and plasmids used in this study.

Strains and plasmids	Relevant features	References
Strains		
DH5α	*E. coli*, α-complementation	Stratagene
KY2347	*E. coli* strain MG1655, Δ(*clpPX-lon*) 1196::*cat*	(Herman *et al.*, 1998)
NTL4	*A. tumefaciens*, Ti-plasmidless derivative of strain C58	(Luo *et al.*, 2001)
WCF47	*A. tumefaciens* containing a non polar internal deletion of *traI*	(Zhu *et al.*, 1998)
Plasmids		
pYC335	*traR* cloned into *EcoRI* and *BamHI* sites of pPZP201	(Chai and Winans, 2004)
pPZP201	broad-host range cloning vector, Sp^R	(Hajdukiewicz *et al.*, 1994)
pCEW1055	Consensus P*traM-lacZ* fusion, Km^R	(White and Winans, 2005)
pCEW250	Consensus *tra box* at P*traI*	(White and Winans, 2007)
pBBR1MCS5	Broad host range vector, Gm^R	(Kovach *et al.*, 1995)
pCEW260	Consensus P*traI-lacZ* fusion, Km^R	(White and Winans, 2007)

DNA manipulations and strain constructions

Recombinant DNA techniques were performed using standard procedures (Sambrook and Russell, 2001). Plasmid DNA was isolated from *E. coli* with QIAprep spin miniprep kits (Qiagen) for DNA sequence analysis. DNA sequences of constructs that were obtained by PCR were verified using automated DNA sequencing (Cornell Biotechnology Resource Center) and analyzed using the LaserGene program (DNASTAR). Plasmids were introduced into *E. coli* by transformation (Sambrook and Russell, 2001) and

into *A. tumefaciens* by electroporation (Cangelosi *et al.*, 1991). *E. coli* strain DH5α was used for all plasmid constructions.

Site-directed mutagenesis

Site-directed mutagenesis of TraR was performed using synthetic overlap extension PCR (Sambrook and Russell, 2001). A 978 bp fragment of plasmid pYC335 was amplified using *Taq* polymerase High Fidelity (Invitrogen). The restriction sites for *EcoR*I and *Sac*II were used to introduce mutated DNA fragments into the wild type gene. For mutations T167S, A168V and E169Q, we used *Eco*RI and *Mfe*I. All oligonucleotides used in this study are listed in Table S2 and were obtained from Integrated DNA Technologies (Coralville, Iowa). Restriction enzymes were obtained from New England Biolabs.

In vivo assays for TraR activity

Bioassays were conducted with derivatives of *A. tumefaciens* strain NTL4 harboring plasmid pCEW260, which carries P*traI-lacZ* reporter or pCEW105, which carries a P*traM-lacZ* reporter. Each strain also contained plasmid pYC335, which expresses P*lac-traR*, or a derivative of pYC335 expressing a *traR* point mutant. Strains were cultured in At minimal medium to an OD_{600} of 0.3 to 0.4. Each was then diluted 20 fold into fresh AT medium containing the indicated concentrations of OOHL, and incubated with vigorous aeration for 8 hours. Assays for β-galactosidase activity were performed as

previously described (Miller, 1972). All experiments were conducted in triplicate and repeated at least three times.

Immunodetection of TraR

The abundance of each TraR protein was determined in parallel with the activity assays described above. A portion of each culture was centrifuged and the cell pellets were resuspended in 5% of their original volume in 1x cracking buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, 0.02% bromophenol blue). Cells were disrupted by boiling for 5 min, cooling to -80°C and boiling for another 5 min. A fraction of each sample was size-fractionated using 12% SDS polyacrylamide gels, and electroblotted onto nitrocellulose membranes (BIORAD). The membranes were blocked using TBS (20 mM Tris pH 7.9, 500 mM NaCl, 0.05% Tween20) with 5% skim milk, and immunodetected in TBS with pre-adsorbed polyclonal anti-TraR rabbit antiserum (Chai and Winans, 2004). Goat anti-rabbit IgG conjugated with alkaline phosphatase (BIORAD) was used as the secondary antibody, and the membranes were stained with BCIP (5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt) and NBT (p-nitro blue tetrazolium chloride) (BIORAD). Westerns were performed with fresh cell lysates for each strain at least three times. Data were analyzed using ImageJ (http://rsb.info.nih.gov/ij/) (Rasband, 2004), and normalized against cross-reacting material in each lane.

Gel mobility shift assays

Clarified cell extracts were used for all gel mobility shift assays. Extracts were prepared from the strain KY2347 (a *clp, lon* mutant) carrying pYC335 or derivatives of it carrying each of the *traR* mutants. Strains were cultured at 28^{O} C in LB broth supplemented with 100 ug/mL spectinomycin to an OD_{600} of 0.2, treated with 500 uM IPTG and 200 nM OOHL, and incubated for an additional 6 hours at 28°C. Cells were then harvested, resuspended in SEDG buffer (Pappas and Winans, 2003), disrupted using a French pressure cell (20,000 psi), and clarified by ultracentrifugation. TraR abundance in each extract was estimated using Western immunoblots, which were analyzed using ImageJ (Rasband, 2004). Equivalent amounts of soluble full-length TraR were added to each binding reaction.

A 247 nucleotide fragment containing the consensus *tra* box sequence was constructed by PCR amplification, using pCEW250 as template and oligonucleotides Ptra-box For and Ptra-box Rev as primers (Table S2). A negative control fragment of 211 bp in length was PCR amplified from pCEW250 using the primers Pcontrol gel shift For and Pcontrol gel shift Rev (Table S2). Both fragments were end-labeled with [γ-^{32}P]dATP (Pelkin Elmer) using T4 polynucleotide kinase (New England Biolabs) and incubated with protein extracts as previously described (Zhu and Winans, 1999). Reactions were size-fractionated in 8% polyacrylamide gels at 4^{O} C as previously described (Pappas and Winans, 2003). Results were analyzed using a Storm

B840 PhosphorImager (Molecular Dynamics). Gel shifts were performed with independent clarified lysates at least twice for each strain.

Intragenic complementation

Bioassays were conducted with derivatives of *A. tumefaciens* strain WCF47 (Zhu *et al.*, 1998) carrying a P*traI-lacZ* fusion in the Ti plasmid. The TraR mutants, W184H, D10N and G123R were cloned in both pPZP201 and pBBR1MCS5 using the restriction sites for *EcoR*I and *BamHI.* Every possible mutant/plasmid combination was tested. Assays for β-galactosidase activity using 100 nM of OOHL were performed as previously described (Miller, 1972).

Structural analyses

SWISSPROT PDB Viewer (Guex and Peitsch, 1997) (http://www.expasy.org/spdbv/) and Protein Explorer (Martz, 2002) (http://proteinexplorer.org) were used to identify all surface residues of the TraR NTD, and to map the point mutants onto the structure of TraR. There are two crystal structures of TraR-OOHL-DNA complexes available from RCSB (http://www.rcsb.org) (accession codes are 1L3L and 1HOM).

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Supplementary information

The supplementary information can be found at the end of this chapter.

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2.7. Supplementary information

Table S1: Summary of Mutant Phenotypes

Mutation	Activity at P*traI*				Activity at P*traM*	Accumulation	DNA binding
	0.1nM	1 nM	10 nM	100 nM	100 nM		
Wild type	(100)	(100)	(100)	(100)	(100)	(100)	(100)
Vector Control	<1	<1	<1	<1	11	<1	<1
Q2E	9	51	50	54	72	40	76
H3K	62	104	135	137	99	NT	NT
D6E	20	15	28	42	78	120	89
D6G	<1	<1	<1	<1	16	102	70
D6N	16	58	86	67	75	53	89
K7R	9	31	28	44	75	78	80
T9V	36	57	83	53	110	99	76
D10N	<1	<1	<1	<1	11	101	109
L11A	6	55	74	97	90	71	75
A13L	<1	1	20	7	104	100	65
I14A	2	12	43	63	72	90	NT
E15K	6	18	31	46	98	45	74
E15Q	6	23	36	56	106	75	94
G16A	6	33	112	132	73	102	NT
D17E	12	11	34	21	70	30	38
E18Q	32	56	67	94	94	108	84
C19A	3	32	83	121	103	102	95
I20V	2	28	57	47	105	70	67
I20W	5	12	38	14	100	49	57
K22E	2	9	41	63	76	101	NT
T23E	10	19	52	67	102	90	71
A26S	34	63	72	84	99	96	NT
D27E	2	34	38	60	107	39	55
D30E	8	36	69	107	105	61	103
H31A	5	29	70	75	86	50	73
F32W	78	76	82	58	100	110	NT
G33A	<1	14	43	70	75	98	76
T35A	37	62	85	114	109	101	NT
H41F	120	49	99	57	73	NT	NT
Q43E	67	56	102	66	76	NT	NT
H44K	5	18	34	42	27	20	23
R45A	43	72	101	77	91	115	79
H46E	11	17	53	64	73	82	NT
T48A	8	21	75	102	75	103	NT
N52D	2	5	71	85	57	92	NT
H54K	<1	4	31	66	61	85	NT
R55A	81	63	75	84	86	97	NT
Q56E	47	79	92	80	98	85	NT
S59A	55	63	76	81	92	102	NT

Table S1 (Continued)

T60V	59	71	73	74	55	91	NT
F62W	23	48	34	52	65	NT	NT
D63N	28	57	65	73	93	NT	NT
K64R	76	74	99	107	84	NT	NT
K65R	65	76	101	109	97	92	NT
F66H	<1	6	28	86	91	84	NT
A68V	84	101	101	102	115	75	NT
L69V	53	55	60	67	84	NT	NT
P71H	<1	<1	<1	8	NT	<10	NT
K74E	<1	6	16	8	36	76	74
K74R	NT	134	NT	143	110	NT	NT
R75E	8	32	41	48	40	88	41
R75K	NT	145	NT	157	92	NT	NT
R77E	<1	<1	2	1	15	77	72
R77K	NT	18	NT	138	105	NT	NT
S78E	12	26	22	28	37	100	103
S78T	NT	137	NT	131	99	NT	NT
R79E	32	48	36	36	46	68	63
R79K	NT	128	NT	145	120	NT	NT
K80E	3	19	24	23	102	92	65
K80R	NT	89	NT	108	80	NT	NT
H81E	76	101	102	78	NT	79	NT
T84A	69	72	104	89	72	106	78
E88Q	45	108	117	135	102	80	97
H89R	141	119	117	145	121	130	98
E90K	7	38	81	93	107	107	NT
R91K	3	27	67	116	69	103	90
P92A	83	87	109	156	81	60	99
T93A	37	78	62	100	97	144	100
S95D	54	74	82	102	100	107	NT
S95T	90	94	103	121	69	115	NT
K96R	36	98	99	126	92	60	92
D97N	41	68	95	99	85	50	108
E98K	<1	14	72	117	83	120	NT
R99K	82	65	47	79	88	NT	115
A100V	8	40	76	96	80	52	NT
D103N	29	30	68	91	95	91	112
H104K	26	45	89	85	59	72	98
S106E	66	65	55	50	82	NT	NT
D107N	46	55	69	61	80	20	50
F108H	13	45	92	105	84	107	NT
G109D	7	32	73	95	85	104	NT
R111K	76	87	94	107	97	102	NT
K119E	29	57	70	86	78	80	NT
K119R	73	77	105	111	NT	NT	NT
N122A	<1	3	22	29	62	61	100

Table S1 (Continued)

N122D	<1	1	10	16	33	35	<10
G123R	<1	<1	<1	<1	35	72	110
F124A	85	116	110	68	NT	72	NT
M125I	22	56	64	104	82	103	NT
D133N	81	81	85	90	89	81	NT
K134H	5	22	55	61	87	68	NT
P135G	32	75	83	90	85	101	NT
V136L	48	71	90	101	82	106	NT
D138N	78	90	89	90	76	82	NT
L139A	64	78	82	108	80	NT	NT
D140N	NT	74	NT	105	120	NT	NT
R141E	65	67	74	79	53	76	104
E142Q	66	67	55	84	73	97	NT
D144N	24	42	98	111	99	118	86
D144R	<1	<1	5	23	47	82	74
A147S	32	47	63	77	87	80	NT
S160T	18	41	65	80	75	119	NT
F161W	22	49	77	119	76	135	NT
L162V	7	38	53	90	71	110	NT
R163Q	38	48	48	87	77	NT	NT
T164A	52	52	95	151	84	NT	NT
T165A	73	75	72	80	96	90	100
P166A	121	54	54	116	89	NT	NT
T167S	50	81	76	101	88	85	NT
A168V	37	72	83	101	94	101	NT
E169Q	29	79	77	81	91	106	NT

NT, not tested

Table S2: Oligonucleotides used in this study.

Oligonucleotides	Sequences
Flanking primers	
pT1	5'-CTCACTCATTAGGCACCCCAG-3'
traRb	5'-GTACAACGTGTAGGGCAACGC-3'
Mutagenic primers	
Q2EF	5'-ATGGAGCACTGGCTGGACAAG-3'
Q2ER	5'-CTTGTCCAGCCAGTGCTCCAT-3'
H3KF	5'-ATGCAGAAGTGGCTGGACAAG-3'
H3KR	5'-CTTGTCCAGCCACTTCTGCAT-3'
D6EF	5'-CACTGGCTGGAAAAGCTGACT-3'
D6ER	5'-AGTCAGCTTTTCCAGCCAGTG-3'
D6GF	5'-CACTGGCTGGGCAAGCTGACT-3'
D6GR	5'-AGTCAGCTTGCCCAGCCAGTG-3'
D6NF	5'-CACTGGCTGAACAAGCTGACT-3'
D6NR	5'-AGTCAGCTTGTTCAGCCAGTG-3'
K7RF	5'-TGGCTGGACAAGCTGACTGAT-3'
K7RR	5'-ATCAGTCAGCTTGTCCAGCCA-3'
T9VF	5'-GACAAGCTGGTTGATCTTGC-3'
T9VR	5'-GCAAGATCAACCAGCTTGTC-3'
D10NF	5'-AGCTGACTAATCTTGCCGCG-3'
D10NR	5'-CGCGGCAAGATTAGTCAGCT-3'
L11AF	5'-CTGACTGATGCTGCCGCGATC-3'
L11AR	5'-GATCGCGGCAGCATCAGTCAG-3'
A13LF	5'-ATCTTGCCCTGATCGAAGG-3'
A13LR	5'-CCTTCGATCAGGGCAAGAT-3'
I14AF	5'-CTTGCCGCGGCCGAAGGCGAT-3'
I14AR	5'-ATCGCCTTCGGCCGCGGCAAG-3'
E15KF	5'-CGCGATCAAAGGCGATGA-3'
E15KR	5'-TCATCGCCTTTGATCGCG-3'
E15QF	5'-CGCGATCCAAGGCGATGA-3'
E15QR	5'-TCATCGCCTTGGATCGCG-3'
G16AF	5'-GCGATCGAAGCCGATGAGTGC-3'
G16AR	5'-GCACTCATCGGCTTCGATCGC-3'
D17EF	5'-ATCGAAGGCGAAGAGTGCATC-3'
D17ER	5'-GATGCACTCTTCGCCTTCGAT-3'
E18QF	5'-GAAGGCGATCAGTGCATCCTG-3'
E18QR	5'-CAGGATGCACTGATCGCCTTC-3'
C19AF	5'-GGCGATGAGGCCATCCTGAAG-3'

Table S2 (Continued)

C19AR	5'- CTTCAGGATGGCCTCATCGCC-3'
I20VF	5'-GATGAGTGCGTCCTGAAGACC-3'
I20VR	5'-GGTCTTCAGGACGCACTCATC-3'
I20WF	5'-GATGAGTGCTGGCTGAAGACC-3'
I20WR	5'-GGTCTTCAGCCAGCACTCATC-3'
K22EF	5'-TGCATCCTGGAGACCGGGCTG-3'
K22ER	5'-CAGCCCGGTCTCCAGGATGCA-3'
T23EF	5'-TCCTGAAGGAAGGGCTGGCG-3'
T23ER	5'-CGCCAGCCCTTCCTTCAGGA-3'
A26SF	5'-ACCGGGCTGAGCGACATCGCCG-3'
A26SR	5'-CGGCGATGTCGCTCAGCCCGGT-3'
D27EF	5'-GGGCTGGCGGAGATCGCCGAC-3'
D27ER	5'-GTCGGCGATCTCCGCCAGCCC-3'
D30EF	5'-GACATCGCCGAACATTTCGGC-3'
D30ER	5'-GCCGAAATGTTCGGCGATGTC-3'
H31AF	5'-ATCGCCGACGCATTCGGCTTC-3'
H31AR	5'-GAAGCCGAATGCGTCGGCGAT-3'
F32WF	5'-GCCGACCATTGGGGCTTCAC-3'
F32WR	5'-GTGAAGCCCCAATGGTCGGC-3'
G33AF	5'-GACCATTTCGCCTTCACCGGC-3'
G33AR	5'-GCCGGTGAAGGCGAAATGGTC-3'
T35AF	5'-TTCGGCTTCGCCGGCTATGC-3'
T35AR	5'-GCATAGCCGGCGAAGCCGAA-3'
H41FF	5'-GCCTACCTTTTCATCCAGCAC-3'
H41FR	5'-GTGCTGGATGAAAAGGTAGGC-3'
Q43EF	5'-CTTCATATCGAGCACAGGCA-3'
Q43ER	5'-TGCCTGTGCTCGATATGAAG-3'
H44KF	5'-CATATCCAGAAGAGGCACATC-3'
H44KR	5'-GATGTGCCTCTTCTGGATATG-3'
R45AF	5'-ATCCAGCACGCGCACATCAC-3'
R45AR	5'-GTGATGTGCGCGTGCTGGAT-3'
H46EF	5'-CAGCACAGGGAGATCACCGC-3'
H46ER	5'-GCGGTGATCTCCCTGTGCTG-3'
T48AF	5'-AGGCACATCGCCGCCGTTAC-3'
T48AR	5'-GTAACGGCGGCGATGTGCCT-3'
N52DF	5'-GCCGTTACCGACTATCACCGC-3'
N52DR	5'-GCGGTGATAGTCGGTAACGGC-3'
H54KF	5'-ACCAACTATAAGCGCCAATG-3'
H54KR	5'-CATTGGCGCTTATAGTTGGT-3'

Table S2 (Continued)

S78TR	5'-GTGCTTCCGGGTCCTCGCGCG-3'
R79EF	5'-GCGAGGTCCGAGAAGCACATC-3'
R79ER	5'-GATGTGCTTCTCGGACCTCGC-3'
R79KF	5'-GCGAGGTCCAAGAAGCACATC-3'
R79KR	5'-GATGTGCTTCTTGGACCTCGC-3'
K80EF	5'-AGGTCCCGGGAGCACATCTTC-3'
K80ER	5'-GAAGATGTGCTCCCGGGACCT-3'
K80RF	5'-AGGTCCCGGAGGCACATCTTC-3'
K80RR	5'-GAAGATGTGCCTCCGGGA CCT-3'
H81EF	5'-TCCCGGAAGGAGATCTTCAC-3'
H81ER	5'-GTGAAGATCTCCTTCCGGGA-3'
T84AF	5'-CACATCTTCGCCTGGTCGGGC-3'
T84AR	5'-GCCCGACCAGGCGAAGATGTG-3'
E88QF	5'-TGGTCGGGCCAGCACGAGCG-3'
E88QR	5'-CGCTCGTGCTGGCCCGACCA-3'
H89RF	5'-TCGGGCGAGCGGGAGCGGCCG-3'
H89RR	5'-CGGCCGCTCCCGCTCGCCCGA-3'
E90KF	5'-CGGGCGAGCACAAGCGGCCGAC-3'
E90KR	5'-GTCGGCCGCTTGTGCTCGCCCG-3'
R91KF	5'-GAGCACGAGAAGCCGACGCTG-3'
R91KR	5'-CAGCGTCGGCTTCTCGTGCTC-3'
P92AF	5'-CACGAGCGGGCGACGCTGTCG-3'
P92AR	5'-CGACAGCGTCGCCCGCTCGTG-3'
T93AF	5'-GAGCGGCCGGCGCTGTCGAAG-3'
T93AR	5'-CTTCGACAGCGCCGGCCGCTC-3'
S95DF	5'-GCCGACGCTGGACAAGGACGAG-3'
S95DR	5'-CTCGTCCTTGTCCAGCGTCGGC-3'
S95TF	5'-CGACGCTGACGAAGGACGAGC-3'
S95TR	5'-GCTCGTCCTTCGTCAGCGTCG-3'
K96RF	5'-ACGCTGTCGCGGGACGAGCGT-3'
K96RR	5'-ACGCTCGTCCCGCGACAGCGT-3'
D97NF	5'-CTGTCGAAGAACGAGCGTGC-3'
D97NR	5'-GCACGCTCGTTCTTCGACAG-3'
E98KF	5'-TCGAAGGACAAGCGTGCCTTC-3'
E98KR	5'-GAAGGCACGCTTGTCCTTCGA-3'
R99KF	5'-AGGACGAGAAGGCCTTCTAT-3'
R99KR	5'-ATAGAAGGCCTTCTCGTCCT-3'
A100VF	5'-GACGAGCGTGTGTTCTATGAC-3'
A100VR	5'-GTCATAGAACACACGCTCGTC-3'

Table S2 (Continued)

R55AF	5'-ACTATCACGCCCAATGGCAAT-3'
R55AR	5'-ATTGCCATTGGGCGTGATAGT-3'
Q56EF	5'-TATCACCGCGAATGGCAATCA-3'
Q56ER	5'-TGATTGCCATTCGCGGTGATA-3'
S59AF	5'-CAATGGCAAGCAACCTACTTC-3'
S59AR	5'-GAAGTAGGTTGCTTGCCATTG-3'
T60VF	5'-TGGCAATCAGTCTACTTCGAC-3'
T60VR	5'-GTCGAAGTAGACTGATTGCCA-3'
F62WF	5'-TCAACCTACTGGGACAAGAAG-3'
F62WR	5'-CTTCTTGTCCCAGTAGGTTGA-3'
D63NF	5'-ACCTACTTCAACAAGAAGTTC-3'
D63NR	5'-GAACTTCTTGTTGAAGTAGGT-3'
K64RF	5'-TACTTCGACCGGAAGTTCGA-3'
K64RR	5'-TCGAACTTCCGGTCGAAGTA-3'
K65RF	5'-TCGACAAGCGGTTCGAAGCG-3'
K65RR	5'-CGCTTCGAACCGCTTGTCGA-3'
F66HF	5'-GACAAGAAGCATGAAGCGCTC-3'
F66HR	5'-GAGCGCTTCATGCTTCTTGTC-3'
A68VF	5'-AGTTCGAAGTGCTCGATCCG-3'
A68VR	5'-CGGATCGAGCACTTCGAACT-3'
L69VF	5'-TCGAAGCGGTCGATCCGGTCG-3'
L69VR	5'-CGACCGGATCGACCGCTTCGA-3'
P71HF	5'-GCGCTCGATCACGTCGTCAAAC-3'
P71HR	5'-GTTTGACGACGTGATCGAGCGC-3'
K74EF	5'-CCGGTCGTCGAACGCGCGAGG-3'
K74ER	5'-CCTCGCGCGTTCGACGACCGG-3'
K74RF	5'-CGGTCGTCAGACGCGCGAGGT-3'
K74RR	5'-ACCTCGCGCGTCTGACGACCG-3'
R75EF	5'-GTCGTCAAAGAGGCGAGGTCCCG-3'
R75ER	5'-CGGGACCTCGCCTCTTTGACGAC-3'
R75KF	5'-GTCGTCAAAAAGGCGAGGTC-3'
R75KR	5'-GACCTCGCCTTTTTGACGAC-3'
R77EF	5'-CAAACGCGCGGAGTCCCGGAAG-3'
R77ER	5'-CTTCCGGGACTCCGCGCGTTTG-3'
R77KF	5'-CAAACGCGCGAAGTCCCGGAAG-3'
R77KR	5'-CTTCCGGGACTTCGCGCGTTTG-3'
S78EF	5'-CGCGCGAGGGAGCGGAAGCAC-3'
S78ER	5'-GTGCTTCCGCTCCCTCGCGCG-3'
S78TF	5'-CGCGCGAGGACCCGGAAGCAC-3'

Table S2 (Continued)

S78TR	5'-GTGCTTCCGGGTCCTCGCGCG-3'
R79EF	5'-GCGAGGTCCGAGAAGCACATC-3'
R79ER	5'-GATGTGCTTCTCGGACCTCGC-3'
R79KF	5'-GCGAGGTCCAAGAAGCACATC-3'
R79KR	5'-GATGTGCTTCTTGGACCTCGC-3'
K80EF	5'-AGGTCCCGGGAGCACATCTTC-3'
K80ER	5'-GAAGATGTGCTCCCGGGACCT-3'
K80RF	5'-AGGTCCCGGAGGCACATCTTC-3'
K80RR	5'-GAAGATGTGCCTCCGGGA CCT-3'
H81EF	5'-TCCCGGAAGGAGATCTTCAC-3'
H81ER	5'-GTGAAGATCTCCTTCCGGGA-3'
T84AF	5'-CACATCTTCGCCTGGTCGGGC-3'
T84AR	5'-GCCCGACCAGGCGAAGATGTG-3'
E88QF	5'-TGGTCGGGCCAGCACGAGCG-3'
E88QR	5'-CGCTCGTGCTGGCCCGACCA-3'
H89RF	5'-TCGGGCGAGCGGGAGCGGCCG-3'
H89RR	5'-CGGCCGCTCCCGCTCGCCCGA-3'
E90KF	5'-CGGGCGAGCACAAGCGGCCGAC-3'
E90KR	5'-GTCGGCCGCTTGTGCTCGCCCG-3'
R91KF	5'-GAGCACGAGAAGCCGACGCTG-3'
R91KR	5'-CAGCGTCGGCTTCTCGTGCTC-3'
P92AF	5'-CACGAGCGGGCGACGCTGTCG-3'
P92AR	5'-CGACAGCGTCGCCCGCTCGTG-3'
T93AF	5'-GAGCGGCCGGCGCTGTCGAAG-3'
T93AR	5'-CTTCGACAGCGCCGGCCGCTC-3'
S95DF	5'-GCCGACGCTGGACAAGGACGAG-3'
S95DR	5'-CTCGTCCTTGTCCAGCGTCGGC-3'
S95TF	5'-CGACGCTGACGAAGGACGAGC-3'
S95TR	5'-GCTCGTCCTTCGTCAGCGTCG-3'
K96RF	5'-ACGCTGTCGCGGGACGAGCGT-3'
K96RR	5'-ACGCTCGTCCCGCGACAGCGT-3'
D97NF	5'-CTGTCGAAGAACGAGCGTGC-3'
D97NR	5'-GCACGCTCGTTCTTCGACAG-3'
E98KF	5'-TCGAAGGACAAGCGTGCCTTC-3'
E98KR	5'-GAAGGCACGCTTGTCCTTCGA-3'
R99KF	5'-AGGACGAGAAGGCCTTCTAT-3'
R99KR	5'-ATAGAAGGCCTTCTCGTCCT-3'
A100VF	5'-GACGAGCGTGTGTTCTATGAC-3'
A100VR	5'-GTCATAGAACACACGCTCGTC-3'

Table S2 (Continued)

D103NF	5'-GCCTTCTATAACCACGCATC-3'
D103NR	5'-GATGCGTGGTTATAGAAGGC-3'
H104KF	5'-TCTATGACAAGGCATCCGAT-3'
H104KR	5'-ATCGGATGCCTTGTCATAGA-3'
S106EF	5'-GACCACGCAGAGGATTTCGGC-3'
S106ER	5'-GCCGAAATCCTCTGCGTGGTC-3'
D107NF	5'-CACGCATCCAACTTCGGCATC-3'
D107NR	5'-GATGCCGAAGTTGGATGCGTG-3'
F108HF	5'-GCATCCGATCATGGCATCCGC-3'
F108HR	5'-GCGGATGCCATGATCGGATGC-3'
G109DF	5'-TCCGATTTCGACATCCGCTCCG-3'
G109DR	5'-CGGAGCGGATGTCGAAATCGGA-3'
R111KF	5'-TCGGCATCAAGTCCGGCATC-3'
R111KR	5'-GATGCCGGACTTGATGCCGA-3'
K119EF	5'-ATACCCATCGAGACCGCCAAC-3'
K119ER	5'-GTTGGCGGTCTCGATGGGTAT-3'
K119RF	5'-ATACCCATCCGGACCGCCAAC-3'
K119RR	5'-GTTGGCGGTCCGGATGGGTAT-3'
N122AF	5'-CAAGACCGCCGCCGGCTTTATGT-3'
N122AR	5'-ACATAAAGCCGGCGGCGGTCTTG-3'
N122DF	5'-TCAAGACCGCCGACGGCTTTAT-3'
N122DR	5'-ATAAAGCCGTCGGCGGTCTTGA-3'
G123RF	5'-ACCGCCAACCGCTTTATGTCG-3'
G123RR	5'-CGACATAAAGCGGTTGGCGGT-3'
F124AF	5'-GCCAACGGCGCCATGTCGATG-3'
F124AR	5'-CATCGACATGGCGCCGTTGGC-3'
M125IF	5'-CAACGGCTTTATCTCGATGTTCA-3'
M125IR	5'-TGAACATCGAGATAAAGCCGTTG-3'
D133NF	5'-ATGGCATCGAACAAGCCGGTG-3'
D133NR	5'-CACCGGCTTGTTCGATGCCAT-3'
K134HF	5'-GCATCGGACCACCCGGTGATC-3'
K134HR	5'-GATCACCGGGTGGTCCGATGC-3'
P135GF	5'-TCGGACAAGGGTGTGATCGAT-3'
P135GR	5'-ATCGATCACACCCTTGTCCGA-3'
V136LF	5'-GACAAGCCGTTGATCGATCTC-3'
V136LR	5'-GAGATCGATCAACGGCTTGTC-3'
D138NF	5'-GCCGGTGATCAACCTCGATCG-3'
D138NR	5'-CGATCGAGGTTGATCACCGGC-3'

Table S2 (Continued)

L139AF	5'-GTGATCGATGCCGATCGGGAG-3'
L139AR	5'-CTCCCGATCGGCATCGATCAC-3'
D140NF	5'-ATCGATCTCAACCGGGAGATC-3'
D140NR	5'-GATCTCCCGGTTGAGATCGAT-3'
R141EF	5'-GATCTCGATGAGGAGATCGAT-3'
R141ER	5'-ATCGATCTCCTCATCGAGATC-3'
E142QF	5'-CTCGATCGGCAGATCGATGCA-3'
E142QR	5'-TGCATCGATCTGCCGATCGAG-3'
D144NF	5'-CGGGAGATCAACGCAGTCGCA-3'
D144NR	5'-TGCGACTGCGTTGATCTCCCG-3'
D144RF	5'-CGGGAGATCCGTGCAGTCGCA-3'
D144RR	5'-TGCGACTGCACGGATCTCCCG-3'
A147SF	5'-GATGCAGTCAGCGCCGCTGCA-3'
A147SR	5'-TGCAGCGGCGCTGACTGCATC-3'
S160TF	5'-GCCCGCATCACATTCCTTCGC-3'
S160TR	5'-GCGAAGGAATGTGATGCGGGC-3'
F161WF	5'-CGCATCTCATGGCTTCGCAC-3'
F161WR	5'-GTGCGAAGCCATGAGATGCG-3'
L162VF	5'-ATCTCATTCGTTCGCACCAC-3'
L162VR	5'-GTGGTGCGAACGAATGAGAT-3'
R163QF	5'-TCATTCCTTCAGACCACCCCT-3'
R163QR	5'-AGGGGTGGTCTGAAGGAATGA-3'
T164AF	5'-TCCTTCGCGCCACCCCTACCG-3'
T164AR	5'-CGGTAGGGGTGGCGCGAAGGA-3'
T165AF	5'-CTTCGCACCGCCCCTACCGCG-3'
T165AR	5'-CGCGGTAGGGGCGGTGCGAAG-3'
P166AF	5'-CGCACCACCGCTACCGCGGA-3'
P166AR	5'-TCCGCGGTAGCGGTGGTGCG -3'
T167SF	5'-ACCACCCCTTCCGCGGAAGAT-3'
T167SR	5'-ATCTTCCGCGGAAGGGGTGGT-3'
A168VF	5'-ACCCCTACCGTGGAAGATGC-3'
A168VR	5'-GCATCTTCCACGGTAGGGGT-3'
E169QF	5'-CTACCGCGCAAGATGCCGCA-3'
E169QR	5'-TGCGGCATCTTGCGCGGTAG-3'

[2]CHAPTER 3

The DNA Dinding Domain of TraR Contains Amino Acid Residues That Increase Protease Susceptibility

3.1. Summary

TraR of *Agrobacterium tumefaciens* is a LuxR-type transcription factor that regulates genes required for replication and conjugation of the tumor-inducing (Ti) plasmid. TraR binds the pheromone 3-oxo-octanoylhomoserine lactone (OOHL) and requires this molecule for activity and for folding into a soluble and protease-resistant conformation. Even after binding to OOHL, TraR is degraded at detectable rates. In this study we show that the N-terminal domain of TraR, which binds OOHL, is more resistant to degradation than the full length protein. Degradation requires sites found on the C-terminal DNA binding domain (TraRCTD). TraRCTD is extremely unstable when expressed alone, and destabilizes fusion proteins containing maltose binding protein or green fluorescent protein. When the C-terminus of GFP was fused to the N-terminus of TraRCTD, the resulting fusion was poorly fluorescent due to proteolysis. We identified residues at two regions of TraR-CTD that contribute to protein degradation. It seems that HslVU protease is involved in the degradation of GFP-TraRCTD. We also performed pulse chase assays

[2]Chapter 3 is a manuscript prepared for submission. Costa, E.D., Chai, Y., and Winans, S.C. The DNA binding domain of TraR contains amino acids that increase protease susceptibility. Figures 3.1, 3.2, 3.3 and Table 3.3 were done by Chai, Y. All the other Figures and tables were done by Costa, E.D.

and found that mutations in TraR-CTD previously shown to accumulate more than the wild-type protein also have an increased half-life. These combined results show that features found in TraRCTD enhance protease susceptibility.

3.2. Introduction

The cellular abundance of any protein is determined by its rate of synthesis, the rate at which cell division dilutes the protein, and the rate of its degradation by cellular proteases (Gottesman, 2003). In eukaryotic cells, protein degradation generally occurs after a protein is polyubiquitinated by a ubiquitin ligase (Glickman and Ciechanover, 2002). These covalently modified proteins then enter the 26S proteasome and are reduced to short peptides. Bacteria do not appear to covalently modify proteins to mark them for proteolysis, but nevertheless can selectively degrade certain proteins via several cytoplasmic proteases. These proteases are multisubunit complexes containing a substrate binding component that binds and unfolds the substrate protein at the expense of ATP, and a protease component whose active site lies within a hollow core of the complex (Gottesman, 1996).

Many proteins are required only at particular times of the cell cycle or in response to particular environmental stimuli and they are removed from the cell by proteolysis at times that they are unneeded or even deleterious (Frank *et al.*, 1996; Jenal and Fuchs, 1998; Turgay *et al.*, 1998; Zhou *et al.*, 2001). At least some of these proteins have evolved features that enhance protease

susceptibility. In general terms, hydrophobic residues that are exposed to the aqueous milieu are thought to act as a signal (Gottesman, 2003). Such a signal also serves to target denatured or misfolded proteins, as correctly folded proteins generally have hydrophobic interiors and hydrophilic exteriors.

Hydrophobic residues on the exterior of a fully folded protein can also serve as a proteolysis signal. These sequences are frequently, though not always, found at the amino or carboxyl terminus of a protein. A particularly well studied example of this is the CtrA protein of *Caulobacter crescentus*. This protein regulates transcription of genes required for the progression through the cell cycle. CtrA accumulates during the G1 phase, regulates almost 100 promoters, and then is degraded by the ClpXP protease (Jenal, 2009). CtrA has two alanine residues at its C-terminus that provide a target for proteolysis (Domian *et al.*, 1997).

The TraR protein of *Agrobacterium tumefaciens* may provide another example of a protein with built-in protease recognition features. TraR is a LuxR-type quorum sensing transcription factor that binds an autoinducer-type pheromone (3-oxo-octanoylhomoserine lactone, OOHL) (Zhu and Winans, 1999). TraR-OOHL complexes bind to the promoters of the Ti plasmid genes required for vegetative replication or for conjugative transfer (Fuqua and Winans, 1996; Fuqua and Winans, 1994; Li and Farrand, 2000; Pappas and Winans, 2003). TraR requires OOHL, not only for activity, but also for solubility and for folding into a protease-resistant form (Zhu and Winans, 1999, 2001). In

the absence of OOHL, apo-TraR is degraded by the Clp and Lon proteases, with a half-life of just 2 minutes (Zhu and Winans, 2001).

TraR-OOHL complexes, while far more stable than apo-TraR, are nonetheless degraded at readily detectable rates, having a half life of approximately 35 minutes in *A. tumefaciens* (Zhu and Winans, 2001). This turnover could play an important role in turning off TraR activity, especially given the fact that TraR appears to bind OOHL irreversibly (Zhu and Winans, 1999). One might expect that when cells with active TraR are diluted from a high to a low cell density, TraR would remain active. However, the degradation of TraR-OOHL complexes seems sufficiently rapid to eventually extinguish expression of its regulon. In the present study, we show that the C-terminal domain of TraR contains sites that destabilize the protein to proteolysis, and have mapped these sites.

3.3. Materials and Methods

Bacterial Strains, plasmids and oligonucleotides

Bacterial strains and plasmids used in this study are described in Table 3.1, while oligonucleotides used for PCR amplification and mutagenesis, obtained from Integrated DNA Technologies (Coralville, Iowa), are described in Table 3.2. *A. tumefaciens* strains were cultured in AT minimal medium at 28°C (Tempé *et al.*, 1977). *Escherichia coli* strains were cultured in Luria broth (LB) or solid medium at 37°C (Miller, 1972). Synthetic OOHL was provided by

A. Eberhard (Cornell University). Antibiotics were added at the following concentrations: 100 µg/mL spectinomycin, 100 µg/mL kanamycin, 200 µg/mL ampicillin and 10 µg/mL chloramphenicol for *E. coli*, and 200 µg/mL spectinomycin and 200 µg/mL kanamycin for *A. tumefaciens*.

DNA manipulations

Recombinant DNA techniques were performed using standard procedures (Sambrook and Russell, 2001). Plasmid DNA was isolated from *E. coli* with QIAprep spin miniprep kits (Qiagen) for DNA sequence analysis. DNA sequences of constructs that were obtained by PCR were verified using automated DNA sequencing (Cornell Biotechnology Resource Center) and analyzed using the LaserGene program (DNASTAR). Plasmids were introduced into *E. coli* by transformation (Sambrook and Russell, 2001) and into *A. tumefaciens* by electroporation (Cangelosi *et al.*, 1991). *E. coli* strain DH5α was used for all plasmid constructions.

Table 3.1. Strains and plasmids used in this study.

Strains and plasmids	Relevant genotypes	Reference
Strains		
DH5α	*E. coli* , α-complementation	Stratagene
KYC55	*Agrobacterium tumefaciens* R10 lacking Ti plasmid	(Cho *et al.*, 1997)
BL21/DE3	*E. coli B* Plac-gene 1 of bacteriophage T7	(Studier *et al.*, 1990)
SG22163	*malP*::*lacI*Q	(Gottesman *et al.*, 1998)
SG22174	SG22163 *clpP*::*cat*	(Gottesman *et al.*, 1998)
SG22186	SG22163 D*lon rcsA51*::*kan*	(Gottesman *et al.*, 1998)
KY2347	MG1655 D(*clpPX-lon*)*1196*::*cat*	(Herman *et al.*, 1998)
KY2966	MG1655 D*hslVU1172*::*tet*	(Herman *et al.*, 1998)
KY2981	MG1655 D(*clpPX-lon*)*1196*::*cat* D*hslVU1172*::*tet sulA2981*	(Herman *et al.*, 1998)
Plasmids		
pHC012	pHC011 digested with EcoRV and KpnI and ligated to pBBR1-MCS5 after digestion with SphI and KpnI, with 3'-end fill-in of the SphI site with the Klenow fragment of DNA polymerase I; Ptac is fused to NdeI-KpnI-ApaI-XhoI-SalI-Bsp106I-ClaI-HindIII-EcoRI-PstI-SmaI-BamHI-SpeI-XbaI-BstXI-SacI; rep-pBBR1 GmR	(Cho *et al.*, 2009)
pHC016	Full length TraR cloned into NdeI and BamHI sites of pHC012. GmR	H. Cho
pHC017	TraR-CTD (171-234) cloned into pRSETA (NdeI and HindIII sites were used). The first amino acid is methionine. AmpR	H. Cho
pKP105	TraR-NTD (1-170) was cloned into the NdeI and HindIII sites of pRSETA. AmpR	K.M. Pappas
pJZ358	TraR cloned into pRSETA	(Zhu and Winans, 1999)
pJBA27	Apr pUC18Not-PA1/0403-RBSII-gfpmut3*-T0-T1	(Andersen *et al.*, 1998)
pYC335	pYC335 traR cloned into EcoRI and BamHI sites of pPZP201	(Chai and Winans, 2004)
pMAL-C2	Expression vector which encodes maltose-binding protein (MBP) under control of Ptac promoter.	New England Biolabs
pCEW180A	T180A substitution of TraR in pJZ335	(White and Winans, 2005)
pCEW187E	V187E substitution of TraR in pJZ335	(White and Winans, 2005)
pCEW201A	K201A substitution of TraR in pJZ335	(White and Winans, 2005)
pCEW204A	S204A substitution of TraR in pJZ335	(White and Winans, 2005)
pCEW208A	K208A substitution of TraR in pJZ335	(White and Winans, 2005)
pCEW210A	R210A substitution of TraR in pJZ335	(White and Winans, 2005)
pCEW216W	F216W substitution of TraR in pJZ335	(White and Winans, 2005)

Table 3.1. continued

pYC107	TraR-CTD from pHC017 cloned into NdeI and HindIII of pHC012	This study
pYC360	GFP-TraR CTD with V187E substitution in TraRCTD	This study
pYC361	Plac-TraR with A186E substitution in TraR. SpecR	This study
pYC362	Plac-TraR with V187E substitution in TraR. SpecR	This study
pYC363	GFP-TraRCTD with K232E and L233S substitutions in TraRCTD	This study
pYC364	GFP-TraRCTD with L233D substitution in TraR-CTD	This study
pYC365	GFP-TraRCTD with I234D substitution in TraRCTD	This study
pEC514	T180A substitution of TraR from pCEW180A cloned into *BbsI* and *HindIII* sites of pJZ358	This study
pEC515	V187E substitution of TraR from pCEW180A cloned into *BbsI* and *HindIII* sites of pJZ358	This study
pEC517	K201A substitution of TraR from pCEW201A cloned into *BbsI* and *HindIII* sites of pJZ358	This study
pEC518	S204A substitution of TraR from pCEW204A cloned into *BbsI* and *HindIII* sites of pJZ358	This study
pEC519	K208A substitution of TraR from pCEW208A cloned into *BbsI* and *HindIII* sites of pJZ358	This study
pEC520	R210A substitution of TraR from pCEW210A cloned into *BbsI* and *HindIII* sites of pJZ358	This study
pEC521	F216W substitution of TraR from pCEW216W cloned into *BbsI* and *HindIII* sites of pJZ358	This study

Table 3.2. Oligonucleotides used in this study

traRCTD F1	5’-GTACGAATTCGCGGAAGATGCCGCA-3’
traRCTD F2	5’-GTACTGCAGAATTCGCGGAAGCTGCCGCA-3’
traRCTD R1 (insertion of two amino acids - SE at the end of TraRCTD)	5’-GTACAAGCTTCACTCTGAGATGAGTTTCCGCC-3’
traRCTD R2 (L233S)	5’-GTACAAGCTTCAGATGGATTTCCGCCG-3’
traRCTD R3	5’-GCTCAGCTAATTAAGCTTCA-3’
traRCTD R4 (sequencing primer)	5’-GCAACCGAGCGTTCTGAACA-3’
traRCTD R5 (K232E)	5’-GTACAAGCTTCAGATGGATTCCCGCCGGATGGC-3’
traRCTD R6 (L233D)	5’-GTACAAGCTTCAGATGTCTTTCCGCCGGAT-3’
traRCTD R7 (I234D)	5’-GTACAAGCTTCAGTCGAGTTTCCGCCG-3’
traRCTD R8 (I185-A186-V187-stop codon)	5’-CGTAAGCTTTCAGACGGCAATCCATCTCAG-3’
traRCTD R9 (I185-A186-stop codon)	5’-CGTAAGCTTTCAGGCAATCCATCTCAGATA-3’
traRCTD R10 (I185-stop codon)	5’-CGTAAGCTTTCAAATCCATCTCAGATAGGT-3’
traRCTD R11 (I185-A186E-V187-stop codon)	5’-CGTAAGCTTTCAGACTTCAATCCATCTCAGATAGG-3’
traRCTD R12 (I185-A186-V187E-stop codon)	5’-CGTAAGCTTTCACTCGGCAATCCATCTCAGAT-3’
TraRCTD-M1 (A186E in full length TraR)	5’-GAGATGGATTGAGGTCGGCAAGA–3’
TraRCTD M2 (V187E in full length TraR)	5’-ATGGATTGCCGAGGGCAAGACGA–3’

Immunodetection of TraR *in vivo*

The abundance of each TraR protein was determined using strain KYC55 containing plasmids pHC012, pHC016, pYC107 or pYC108. The strains were cultured on 10 ml of AT medium supplemented with appropriate antibiotics, 500 µM IPTG and 250 nM OOHL. When cultures reached an OD600 of 0.5, they were centrifuged and the cell pellets were resuspended in 5% of their original volume in 1x cracking buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, 0.02% bromophenol blue). Cells were disrupted by boiling for 5 min, cooling and boiling for another 5 min. A fraction of each sample was size-fractionated using 15% SDS polyacrylamide gels, and electrophoretically transfered onto nitrocellulose membranes (BIORAD). The membranes were blocked using TBS (20 mM Tris pH 7.9, 500 mM NaCl, 0.05% Tween 20) with 5% skim milk, and immunodetected in TBS with pre-adsorbed polyclonal anti-TraR rabbit antiserum (Chai and Winans, 2004). Goat anti-rabbit IgG conjugated with alkaline phosphatase (BIORAD) was used as the secondary antibody, and the membranes were stained with BCIP (5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt) and NBT (p-nitro blue tetrazolium chloride) (BIORAD). Westerns were performed with fresh cell lysates for each strain at least three times.

OOHL sequestration assay by TraR in whole cells

A. tumefaciens strains KYC55(pYC107), KYC55(pYC108) and KYC55(pHC016) were used for OOHL sequestration assays as previously

described (Chai and Winans, 2004). All assays were performed twice with independent cultures.

TraR stability in *E. coli*

The measurement of TraR turnover was performed using BL21/DE3(pKP105), and BL21/DE3(pJZ358) to compare the stability of TraR-NTD and full length TraR. DH5α(pMAL-C2), DH5α(pYC338), DH5α(pYC339) were used to compare the stability of MBP with the fusion proteins MBP-TraRCTD and MBP-TraRCTD+SE. For the experiments with the mutants of TraR which are more stable than wild type TraR, the turnover of the protein was determined using strain BL21/DE3(pJZ358) (Zhu and Winans, 1999) or derivatives of pJZ358 carrying *traR* mutants. The experiments were performed as described previously (Zhu and Winans, 2001).

Overexpression of MBP-TraRCTD

Overnight stationary-phase cultures of DH5α(pMAL-C2) and DH5α(pYC338) were diluted 50-fold on LB with 400 µg/mL ampicillin and incubated at 37°C to an OD_{600} of 0.4 before induction with 1 mM IPTG. The cultures were incubated until an OD_{600} of 1, and the bacterial cells were harvested by centrifugation. The cells were resuspended in TEDG buffer (200 mM NaCl, 0.1% DTT and 0.1% protease inhibitor cocktail from Sigma-Aldrich) and lysed using a French pressure cell. The crude extract was separated into soluble and pellet fractions by ultracentrifugation at 45K for 30 minutes. Samples were loaded in 10% SDS-polyacrilamide gel.

Random mutagenesis PCR

Random mutagenesis by error-prone PCR was performed using the primers TraRCTDF2 and TraRCTD R3 and the plasmid pYC350 (GFP-TraRCTD) as a template. The PCR products and the plasmid pJBA113 were digested with *Pst*I and *Hin*dIII. The transformants were selected for increased fluorescence compared to wild type under UV light. The candidates were sequenced using primerTraRCTD R4 and the fluorescence was quantified using Synergy HT from BioTek. The fluorescence intensity values were normalized by the OD_{600} to compensate for the variations in cell densities.

Site-directed mutagenesis

Site-directed mutagenesis of TraR was performed using synthetic overlap extension PCR (Sambrook and Russell, 2001). The plasmid pYC350 was used as a template and amplified using *Taq* polymerase High Fidelity (Invitrogen). The restriction sites for *Pst*I and *Hin*dIII were used to introduce mutated DNA fragments into the wild type gene. The forward primer TraRCTD-F1 and reverse primers (R1, R2, R5, R6, R7, R8, R9, R10, R11 and R12) were used to amplify TraR-CTD fragment with various substitutions (Table 3.2). Primers traRCTD-M1 and traRCTD-M2 were used to introduce site directed mutations (A186E and V187E) into full-length TraR using pYC335 as a template.

The fluorescence of each TraR allele, performed in DH5α (pYC350) or derivatives of pYC350 carrying each of the *traR* mutants, was determined

using synergy HT from BioTek. The fluorescence intensity values were normalized by the OD_{600} to compensate for the variations in cell densities.

Protease deficient strains

TraR turnover rates and accumulation were compared in the following protease-deficient strains of *E. coli*: SG22163 (*malP*::*lacI*Q), SG22174 (SG22163 *clpP*::*cat*), SG22186 (SG22163 D*lon rcsA51*::*kan*) (Gottesman *et al.*, 1998), KY2347 (MG1655 D(*clpPX-lon*)*1196*::*cat*), KY2966 (MG1655 D*hslVU1172*::*tet*), and KY2981 (MG1655 D(*clpPX-lon*)*1196*::*cat* D*hslVU1172*::*tet sulA2981*) (Herman *et al.*, 1998). To quantitate TraR-CTD accumulation, the protease deficient strains containing the plasmid pYC350, which contains a fusion protein GFP-TraRCTD, were cultured in LB broth with appropriate antibiotics and IPTG to mid-logarithmic phase at 28°C. The fluorescence was measured using synergy HT from BioTek and normalized by the OD_{600} to compensate for the variations in cell densities.

3.4. Results

TraR(1-170) is more stable than full length TraR

We have previously found that TraR-OOHL complexes are detectably degraded in *Agrobacterium tumefaciens*, with a half-life of approximately 35 minutes (Zhu and Winans, 1999). It was not clear whether particular parts of the protein caused this susceptibility. In the present study, we constructed three plasmids expressing P*tac- traR* fusions, one expressing full length TraR, another expressing only the amino terminal domain of TraR (TraRNTD) (amino acid residues 1-170), and a third expressing only the carboxyl terminal domain of TraR (TraRCTD) (amino acid residues 171-234). The transcription and translation signals of all three genes are derived from the vector and are therefore identical. These plasmids were introduced into *A. tumefaciens* strain KYC55, which lacks a Ti plasmid, cultured to late log phase, and analyzed for TraR content by western immunoblotting.

The strain expressing TraRNTD (1-170) accumulated a protein that was detected by the antiserum (Fig. 3.1, lane 5). This protein had a mass of approximately 19 Kda, in close agreement with the expected mass of this fragment (19.27 Kda). As expected, the abundance of this protein was far greater in the presence of OOHL than in its absence (Fig 3.1, lanes 4 and 5). The strain expressing full length TraR accumulated a protein of 27 Kda which has a mass identical to that of full length TraR (26.67 Kda) (Fig. 3.1, lane 7). The protein was not observed in the absence of OOHL (Fig 3.1, lanes 6).

Significantly, the full length protein observed at Fig. 3.1, lane 7 appeared to be less abundant than the TraRNTD(1-170) expressed from the truncated gene (Fig. 3.1, lane 5).

We also used a strain expressing TraRNTD without the alpha-helix 9 (145-162) which was previously shown to be important for dimerization (Pinto and Winans, 2009; Zhang *et al.*, 2002). TraRNTD lacking alpha helix 9 is very unstable when overexpressed in *A. tumefaciens* from western blot (data not shown). The alpha-helix 9 is directly involved in dimerization and it was previously shown that dimerization enhances resistance of TraR to cytoplasmic proteolysis (Pinto and Winans, 2009).

The *A. tumefaciens* strain expressing the TraRCTD(171-234) did not accumulate detectable levels of the corresponding TraR fragment with an expected size of 7 Kda (Fig 3.1, lanes 2 and 3). Similarly, *E. coli* strain DH5α (pYC107), which contains a P*tac-traR* (171-234) fusion, failed to express detectable levels of the fragment, even when using immunodetection assays (data not shown). We also constructed a PT7-*traR*(171-234) fusion, expressed it using strain BL21/DE3, but failed to detect the fragment immunologically or using conventional protein stains (data not shown). These findings suggest that the TraRCTD(171-234) fragment is extraordinarily unstable to proteolysis, although the formal possibility remained that our polyclonal antiserum did not detect any epitopes present on this fragment.

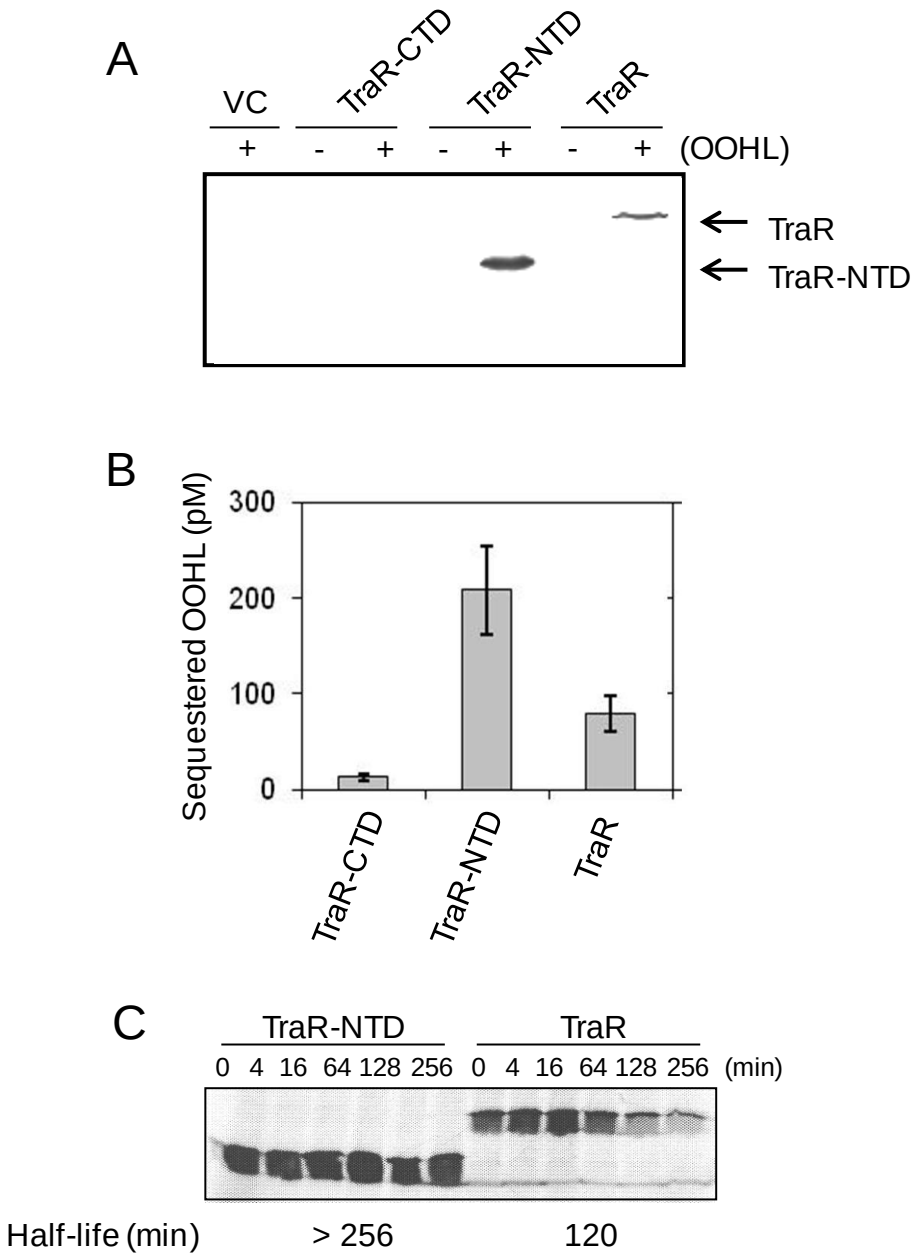

Figure 3.1. N-terminal domain of TraR is more stable than the full-length TraR. (A) Western immunoblot of *A. tumefaciens* KYC55(pHC012) that does not contain TraR (lane 1), KYC55(pYC107) which express TraRCTD (lanes 2 and 3), KYC55(pYC108) which express TraRNTD (lanes 4 and 5) or KYC55(pHC016) which express full length TraR (lanes 6 and 7). (B) OOHL sequestration assays of KYC55(pYC107), KYC55(pYC108) and KYC55(pHC016). (C) Pulse-chase experiments of *A. tumefaciens* KYC55(pYC108) which contains TraRNTD and KYC55(pHC016) which contains full length TraR.

We used a second method to quantify the accumulation of TraR-OOHL complexes. We measured the sequestration of exogenous OOHL by whole cells that express full length TraR, TraRNTD(1-170), and TraRCTD(171-234). We cultured the three strains described above in the presence of OOHL, then washed the cells five times, extracted the OOHL using an organic solvent, and quantified the extracted OOHL using an *A. tumefaciens* bioassay strain. As expected, the strain that expressed TraRCTD(171-234) did not sequester OOHL above background levels (data not shown), while the strains expressing full length TraR or TraRNTD(1-170) sequestered readily detectable amounts. Of these, the strain expressing TraRNTD(1-170) sequestered approximately 2.5 times more OOHL than the strain expressing full length TraR (Fig. 3.1B). These data are supported by the western immunoblots described above, and indicate that TraRNTD(1-170) accumulates to higher levels than full length TraR.

The greater abundance of TraRNTD(1-170) than full length TraR suggests that TraRNTD might be more stable to proteolysis. To test this directly, we expressed both proteins by using the T7 promoter, and performed pulse chase experiments using radiolabeled methionine. Full length TraR had a half life of approximately 120 minutes in *E. coli* (Fig. 3.1C), in reasonable agreement with previous measurements in this experimental system (Zhu and Winans, 2001). In contrast, TraRNTD(1-170) was not detectably degraded over a period of 4 hours.

TraRCTD destabilizes MBP and GFP fusion proteins

As described above, the TraRCTD appeared to destabilize the protein. When the C-terminal domain of TraR (TraR-CTD) was overexpressed in either *E. coli* or *A. tumefaciens*, it was never detectable even by western immunoblot (data not shown). To determine whether it could destabilize unrelated proteins, we constructed a translational fusion between TraRCTD and either maltose binding protein (MBP) or green fluorescent protein (GFP). When overexpressed in *E. coli,* the MBP-TraR(171-234) fusion protein accumulated to lower levels and was less soluble than MBP-LacZα (produced by the vector, Fig. 3.2A). The fusion protein was also degraded more rapidly than MBP-LacZα in pulse labeling experiments (Fig. 3.2B). We made a similar fusion containing two additional amino acids, a serine and a glutamate, at the very end of TraRCTD. The addition of these two amino acids increased the accumulation of the fusion protein indicating that the amino acids at the C-terminal end of the protein (Leu-Ile) might be involved in the destabilization of the fusion protein.

Similar results were obtained using a GFP-TraR(171-234) fusion (3.3A). This fusion was expressed in *E. coli* using a P*lac* promoter. A strain expressing this fusion showed approximately 4-fold lower levels of fluorescence than a strain expressing a GFP control, indicating that TraR(171-234) increased proteolysis of the fusion protein, or, conceivably, that it impaired the correct folding of the fusion (Fig. 3.3A).

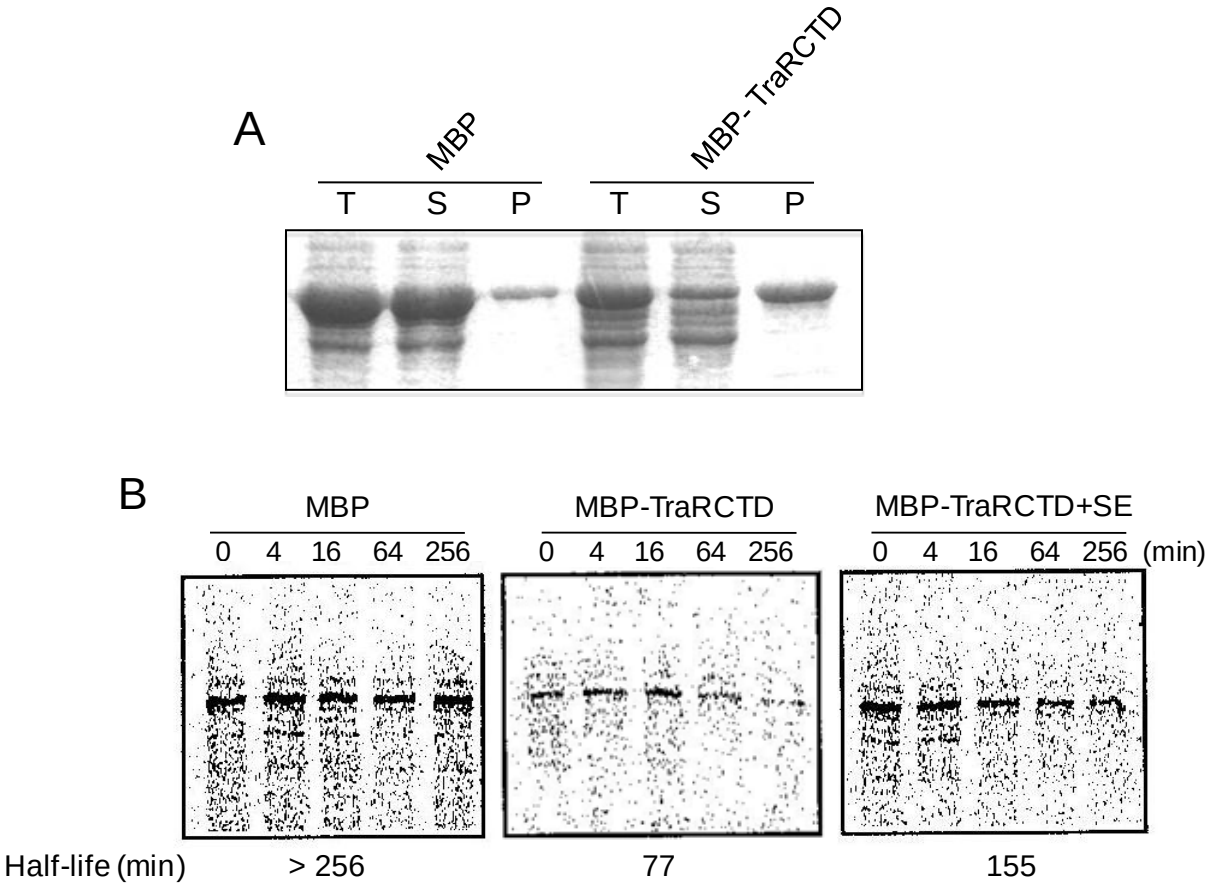

Figure 3.2. C-terminal domain of TraR bears proteolytic signals. (A) Overexpression of DH5α(pMAL-C2) expressing MBP-LacZα and DH5α(pYC338) expressing the fusion protein MBP-TraRCTD. Letters T, S and P represent total, soluble, and pellet fractions of the cell lysates, respectively. The SDS-PAGE was stained with Coomassie brilliant blue dye. (B) Pulse-chase experiment comparing DH5α(pMAL-C2) which contains MBP-LacZα, DH5α(pYC338) which contains the fusion protein MBP-TraRCTD and DH5α(pYC339) which contains the fusion protein TraRCTD with two additional amino acids, Serine and Glutamate (SE), at the end of TraRCTD. Radiolabel was quantified using a Storm Phosporimager. Calculated half-lives are indicated at the bottom of the figure.

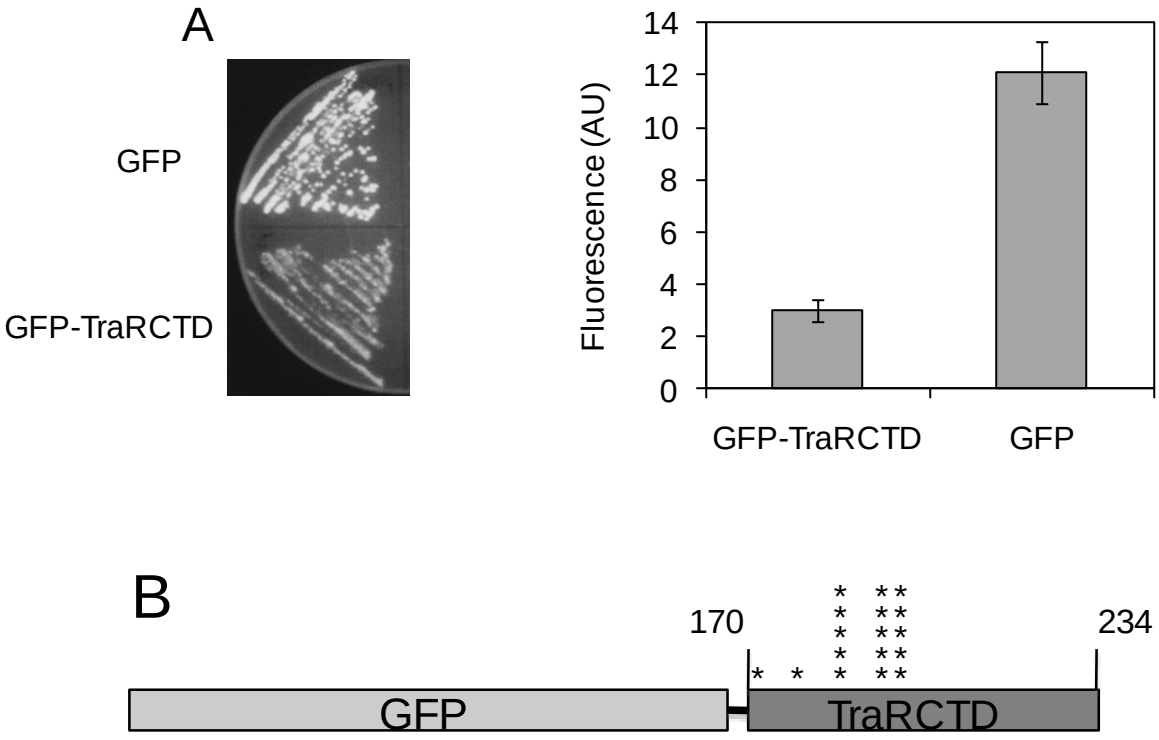

Figure 3.3. Fluorescence of GFP-TraRCTD fusion protein and genetic screen for amino acid residues in TraR-CTD that contain proteolytic signals. (A) A fusion protein between GFP and TraR-CTD is much less fluorescent compared to GFP itself. On the left, *E. coli* colonies expressing either GFP (upper) or GFP-TraRCTD fusion proteins (lower). On the right, fluorescence was measured from the same cells shown on the left. (B) Random mutagenesis of GFP-TraRCTD to search for mutations in *traRCTD* that might increase fluorescence activity of the mutant fusion protein. Stars (*) represent nonsense mutations in *traRCTD* that cause both truncation of the GFP-TraRCTD fusion protein and increased fluorescence of the mutant protein.

Identification of TraR-CTD residues that enhance proteolysis

The GFP-TraRCTD(171-234) fusion protein described above was mutagenized using random mutagenesis PCR and screened for variants showing increased levels of fluorescence. We had hoped to isolate missense mutations that would resist proteolysis better than the parent fusion. To our surprise, all mutants that were found by this screen had nonsense codons within the TraR portion of the fusion protein (Fig. 3.3B).

Of the 57 codons encoded in the TraRCTD(171-234), only 17 can be converted to a stop codon by single base change (Table 3.3). Of these 17 codons, we recovered stop codons at only 5 positions, one each at codons 173 and 177 (numbering from the start codon of full length TraR), and five stop codons at each of the codons 181, 183, and 184. All of these mutations lay near the N-terminus of the TraR(171-234) portion of the fusion protein (Fig. 3.3B). The fact that nonsense mutations were never recovered at any codon downstream of codon 184 provided suggestive evidence that a protease recognition motif could lie fully or partly downstream of this codon. Codons 184-187 encode the hydrophobic residues Trp-Ile-Ala-Val. A stop codon at position 188 would therefore create a C-terminus with four hydrophobic residues that might destabilize the protein.

Table 3.3. Distribution of identified nonsense mutations in GFP-TraRCTD stable variants among the 17 codons that can be converted to a stop codon by single base change.

TraR amino acid residues*	Occurrence of Independent Nonsense Mutations
W173	1
K177	1
E178	0
Y181	5
R183	5
W184	5
K189	0
E192	0
I194	0
E198	0
K201	0
Y202	0
K208	0
E211	0
K214	0
K221	0
R230	0

* Amino acid residues shown here are the ones in the C-terminal domain of TraR that can generate nonsense mutations with single nucleotide change.

In an effort to more finely dissect this possible protease recognition site, we used site-directed mutagenesis to construct the fusions GFP-TraR(170-185), GFP-TraR(170-186) and GFP-TraR(170-187). These fusions end at Ile185, Ala186, and Val187, respectively. GFP-TraR(170-185) had relatively high levels of fluorescence (Fig. 3.4), suggesting that this fusion protein was relatively stable. In contrast, GFP-TraR(170-186) and GFP-TraR(170-187) were far less fluorescent, suggesting that Ala186 and Val187 were required for a protease recognition motif (PRM). We then mutated the gene encoding GFP-TraRCTD(170-187) to make its C-terminus more hydrophilic. We individually altered Ala186 and Val187 both to glutamate residues. Both mutations restored fluorescence to levels comparable to the GFP-TraR(170-186) (Fig. 3.4).

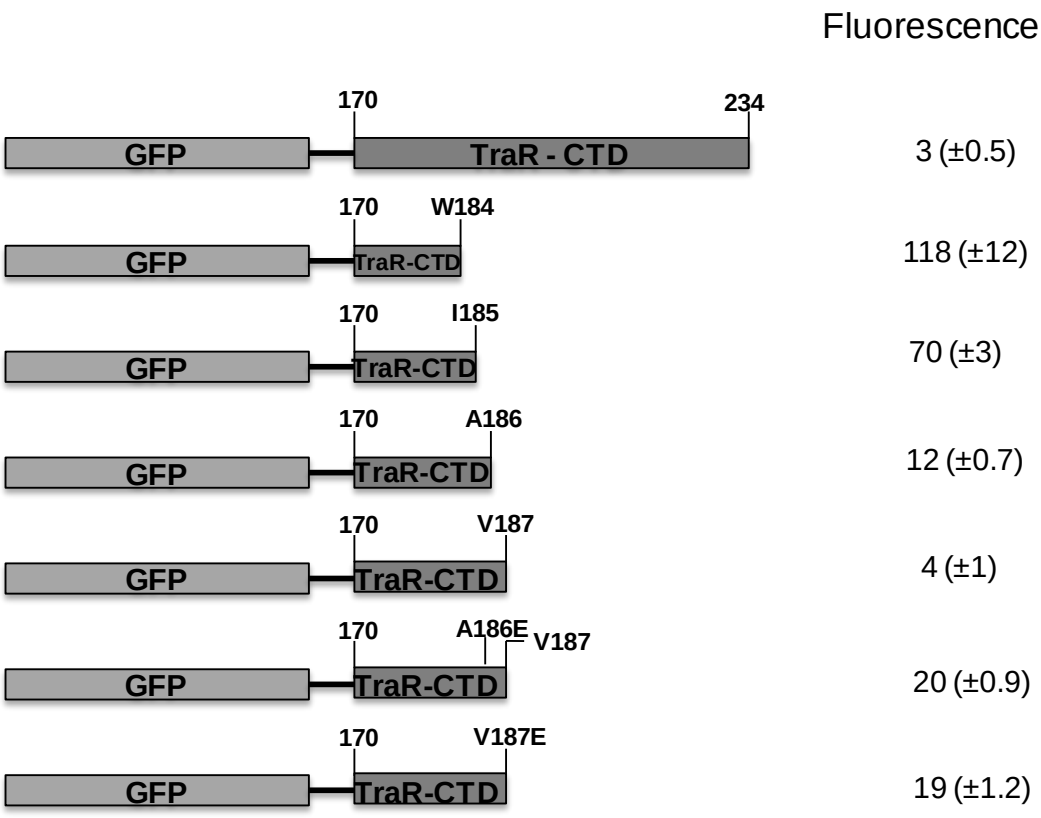

Figure 3.4. Fluorescence Intensity of GFP-TraR(CTD) variants bearing truncations and substitutions in the first protease recognition motif (PRM).

We then determined whether mutations A186E or V187E would stabilize a GFP-TraR that includes the native C-terminus of TraR. These mutations were introduced into a gene expressing GFP-TraRCTD. In the full-length fusion protein, mutation A186E did not increase fluorescence, while V187E caused a modest increase (Fig. 3.5). These findings suggested the existence of one or more additional protease recognition motifs downstream of codon 187. In an effort to identify such a region, we carried out a second round of mutagenesis of a fragment containing the A186E mutation, and screened for elevated fluorescence. One colony was identified that reproducibly showed greater fluorescence than its parent. This gene had a new mutation at codon 233, from Leu to Asp (L233D). This mutation increased the fluorescence of the A186E mutant 4.5 fold. We introduced the L233D mutation into the fusion gene that contained V187E mutation. The resulting fusion protein containing the V187E and L233D mutations also displayed around 4.5 fold increase in the level of fluorescence (Fig. 3.5). The combined point mutations in both the first PRM(V187E) and the second PRM(L233D) gave a higher fluorescent activity compared even to that of V187E (truncated). These data suggest that the TraR-CTD may contain two protease recognition motifs, one that includes residues 184-187, and one at or near the extreme C-terminus of the protein.

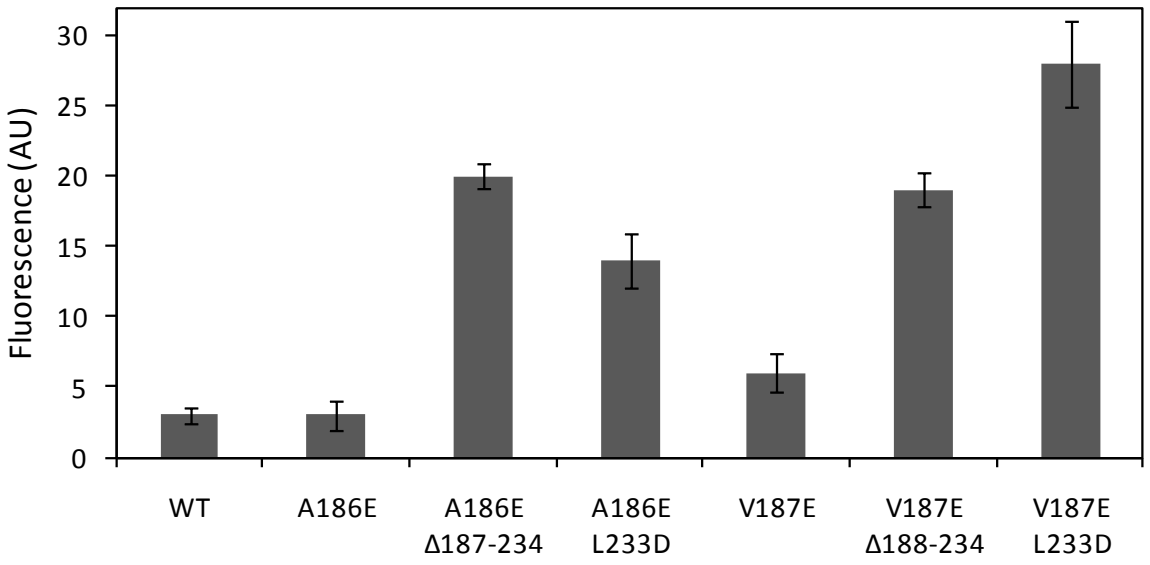

Figure 3.5. Fluorescent activities from *E. coli* cells expressing different GFP-TraRCTD variants.

We then carried out a similar search for the second PRM, the results from which were described in Fig. 3.6. When we added two amino acids at the end of TraRCTD, the fluorescence increased almost 8 fold, suggesting that the amino acids at the end of TraRCTD might be part of a second PRM. The increase in stability by adding two additional amino acids at the end of TraRCTD was also observed in the fusion protein MBP-TraRCTD (Fig. 3.2B). We individually altered amino acids L233 and I234, both to aspartate residues. Both mutations increased the levels of fluorescence when compared to wild type TraRCTD (Fig. 3.6).

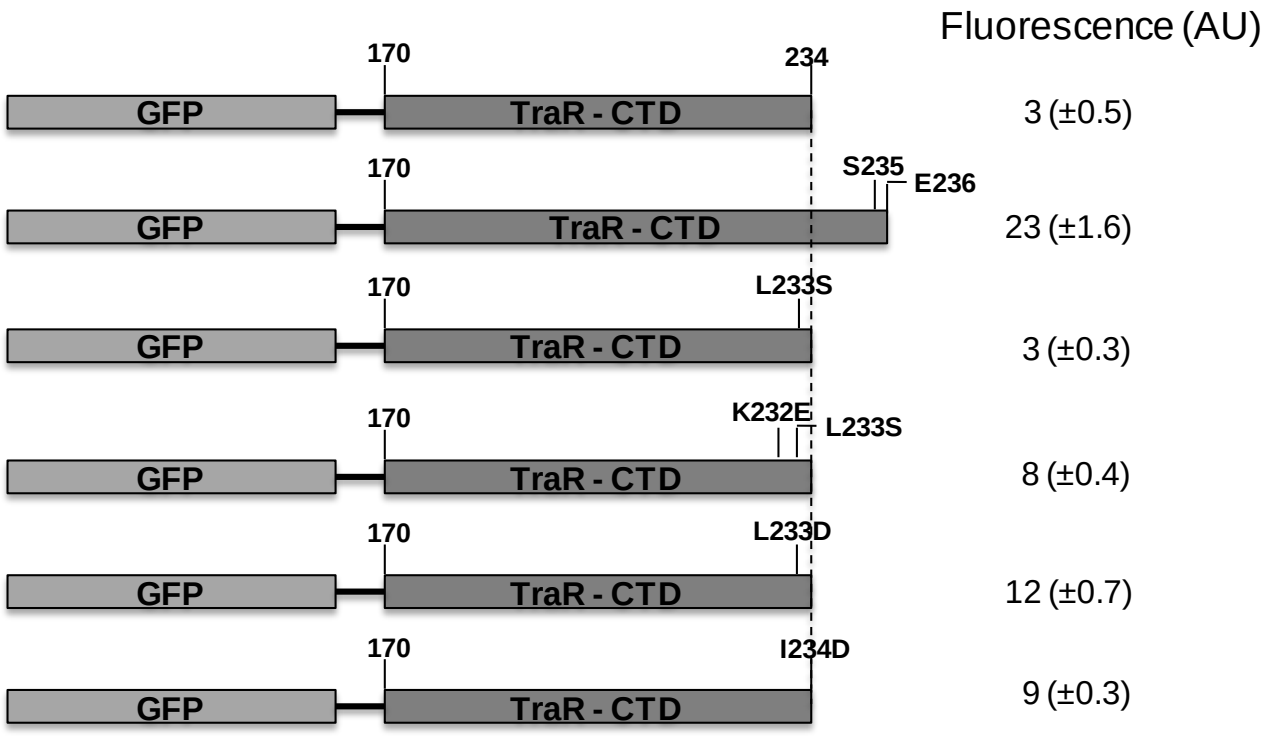

Figure 3.6. Fluorescence activities of GFP-TraR(CTD) variants bearing mutations and substitutions in the second protease recognition motif (PRM).

As shown in Table 3.3, Fig 3.4, Fig 3.5 and Fig 3.6, we discovered two putative protease recognition motifs (183RWIAV187) and (232KLI234) that when removed by truncation or by point mutation, increased the fluorescence of the fusion protein GFP-TraRCTD. These two PRMs are highlighted in the TraRCTD structure in Fig 3.7.

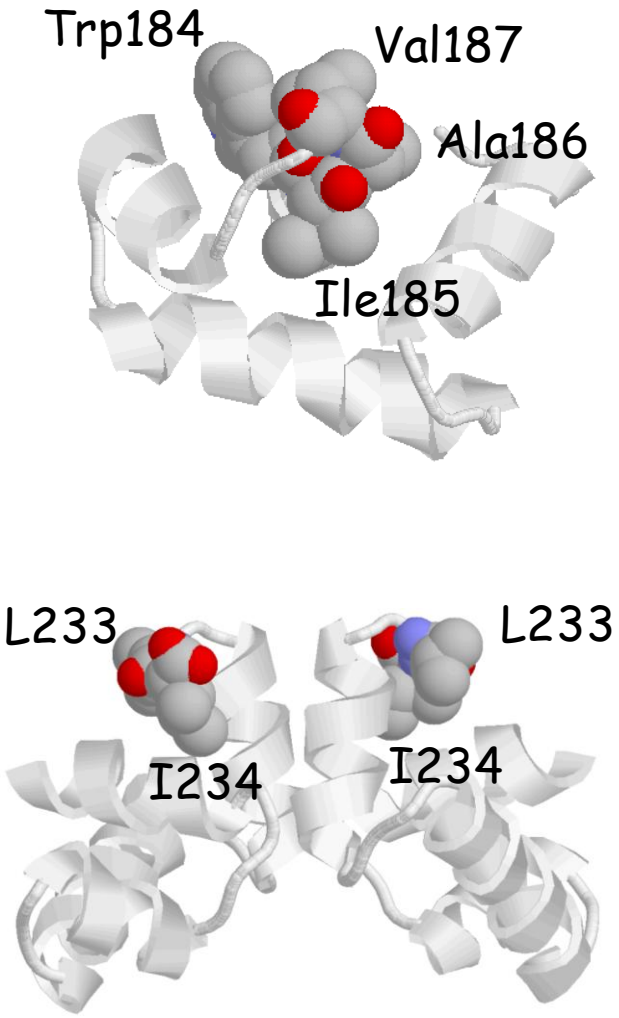

Figure 3.7. Structure of TraR-CTD with highlighted regions representing the first putative protease recognition motif (183-RWIAV-187) and the second recognition motif (233-LI-234) in TraR-CTD.

Clp, Lon and HslVU cause proteolysis of GFP-TraRCTD

Agrobacterium tumefaciens has orthologous genes of several *E. coli* proteases, including ClpP, ClpA, ClpS, ClpX, ClpB, Lon, HslV, HslU, HflX, HflC and HflK (Goodner *et al.*, 2001; Wood *et al.*, 2001). A model for protease function is described in Fig. 3.8.

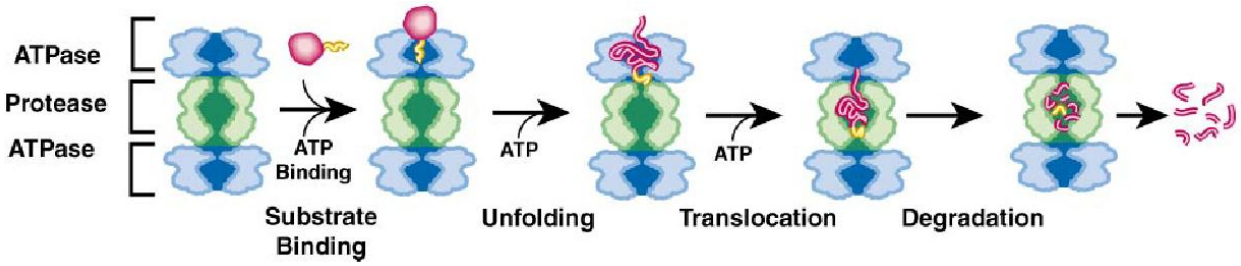

Figure 3.8. Model of Clp protease function. A specific sequence in the substrate (shown in yellow) is recognized the ATPase domain of the protease machinery. The substrate is unfolded with the hydrolysis of ATP and the unfolded substrate is translocated to the proteolytic core, where it is degraded yielding short peptides. Examples of ATPase: ClpA, ClpX, HslU and Lon. Examples of protease: ClpP, HslV and Lon. Figure from (Gottesman, 2003).

We tested protease-deficient *E. coli* strains for fluorescence of the fusion protein GFP-TraRCTD in comparison with GFP. The single mutations in *clpP* or *lon* did not detectably increase the fluorescence of either GFP-TraRCTD or GFP (Fig. 3.9). In contrast, a single mutant in *hslV-hslVU* and a *clpP-clpX*, *lon* double mutant showed an increase in fluorescence for the fusion protein GFP-TraRCTD. The fluorescence increased 5 fold in the *hslVU* cells and 3.5 fold in the *clpPX* and *lon* double mutant compared to the wild type cells (Fig. 3.9). The fluorescence of the GFP also increased in the *hslVU* mutant, but less than 2 fold. This indicates that HSLVU protease might have

some effect on the stability of GFP. Our results show that ClpPX, Lon and HslVU proteases are involved in the degradation of GFP-TraRCTD.

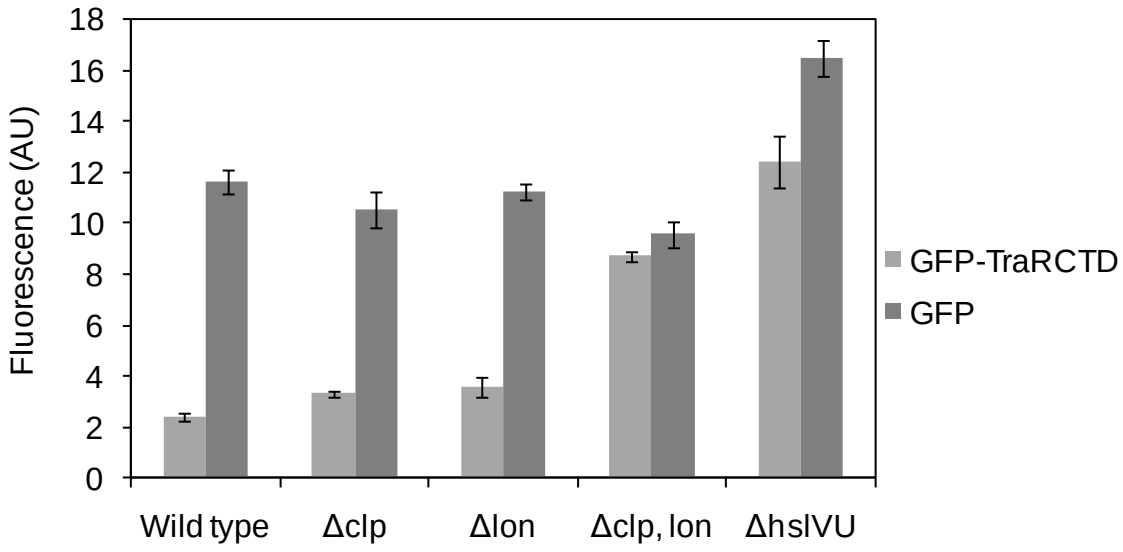

Figure 3.9. *E. coli* wild type and protease deficient strains expressing either GFP or GFP-TraRCTD fusion proteins.

Other amino acids of TraR-CTD decrease protein stability

In a previous study in our laboratory, mutations in amino acids located at the TraRCTD were found to accumulate to higher levels than the wild type TraR, as evaluated by western blots using full length TraR (White and Winans, 2005). This provides further evidence that the wild type sequences at these positions may contribute to the instability of the protein. To test this hypothesis, we performed pulse chase experiments and found that the mutations that increased the accumulation of TraR also increased its half life (Fig. 3.10).

The half life of TraR was 212 minutes at the tested conditions in the presence of OOHL and 2 minutes in the absence of OOHL. This result is in agreement with previous results from our laboratory performed under the same conditions (Pinto and Winans, 2009). The half life of the TraR protein with mutant T180A increased to 309 minutes. It is important to note that this amino acid contacts amino acid W184 of the same TraR subunit and, interestingly, it is in the same α-helix as amino acids in the first protease recognition motif reported in the previous sections.

TraR V187E has a half life of 407 minutes, almost two times longer than the wild type. In a previous study, this amino acid was reported to be involved in the contact between TraR and the RNA polymerase (White and Winans, 2005). It also belongs to the first protease recognition motif, described in the previous sections. The other five mutants that accumulate more than wild type are in the TraR DNA recognition helix. Three of them are hydrophilic, K201, R208 and K210 and the other two are hydrophobic, S204A and F216. All of them have a longer half life than wild type TraR (Fig. 3.10).

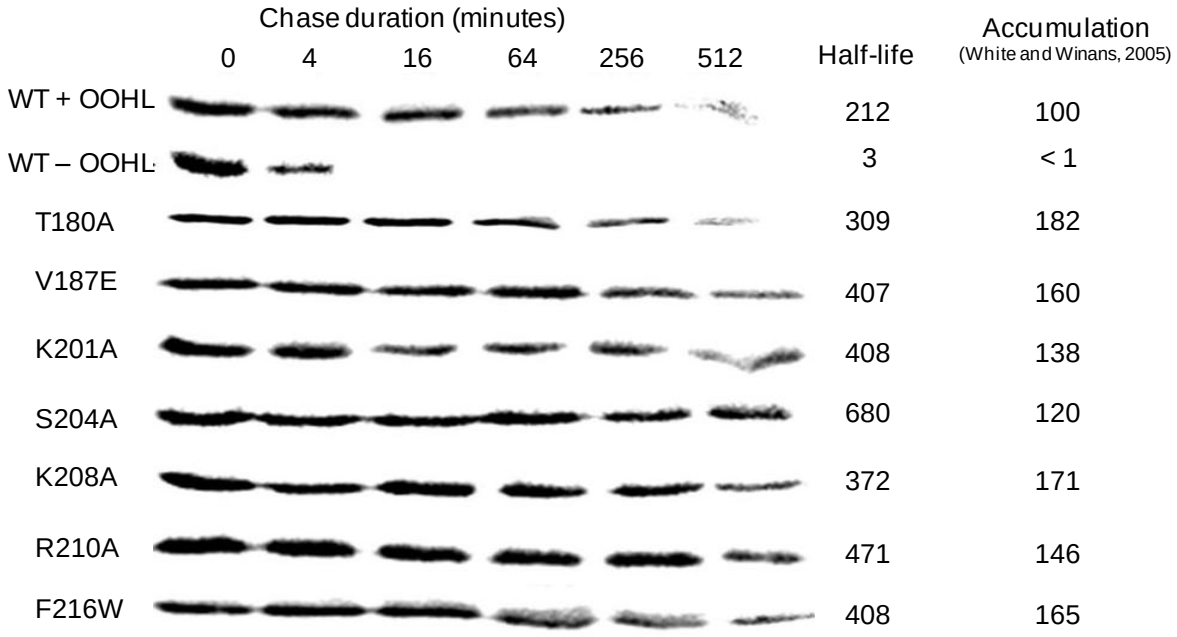

Figure 3.10. Pulse chase of TraR protein with mutations in amino acids of the carboxi-terminal domain that were previously shown to accumulate to higher levels than the wild type protein (White and Winans, 2005). In the figure we show the accumulation and the half-life of each mutant in full lengthTraR.

3.5. Discussion

In this study, we have shown that the two domains of TraR play antagonistic roles in the stability of the protein. TraRNTD(1-170) was not detectably degraded over four hours, while TraRCTD(171-234), did not detectably accumulate, even when driven by the extremely powerful T7 promoter. Clearly, the stability of the full length protein is more similar to its N-terminal domain than its C-terminal domain, suggesting that the N-terminal domain may assist the latter in folding. There are several examples of intra-protein folding chaperones (Fox and Waugh, 2003; Gegg *et al.*, 1997; LaVallie *et al.*, 2003; Nakamura and Iwakura, 1999; Pelletier *et al.*, 1998). Of course, all folding still requires OOHL, without which the full length protein and the NTD are both extremely unstable to proteolysis (Zhu and Winans, 1999, 2001).

Evidence was presented that TraR residues W184, I185, A186, V187 and L233 could destabilize TraR, possibly in conjunction with neighboring residues. Of these, I185, A186 and L233 are sequestered by the opposite subunit of a TraR dimer. They would be surface exposed in a TraR monomer or in a TraR dimer whose two C-terminal domains were dissociated. In contrast, W184 and V187 are exposed to solvent in the TraR dimer. These residues were previously found to be essential for positive control, and mutations at this position inhibit activation of target promoters *in vivo* (Costa et al., 2009; Qin et al., 2009; White and Winans, 2005). These studies suggested that these two residues make direct contact with RNA polymerase, and if so, might be protected from proteolysis by RNA polymerase. It therefore seems

possible that TraR monomers would present several protease-sensitive amino acids, while TraR dimers would present fewer residues, and TraR-RNA polymerase complexes would present none.

The binding to DNA seems to have an effect on the stability of TraR as well. Our results indicate that residue R210, which makes direct contact with DNA (White and Winans, 2007) could also destabilize TraR. Neighboring amino acids, including K208 previously reported to contribute to the affinity of TraR to DNA (White and Winans, 2007), also seem to play a role in TraR stability. When TraR is bound to the DNA, these residues are protected from proteolysis. Therefore, TraR would be more stable in cells if it is in the active form, as a dimer, and if it is bound to DNA.

In earlier studies, the surface of TraR was systematically mutated in a search for residues involved in the contact with the RNA polymerase (Costa *et al.*, 2009; White and Winans, 2005). Of the 60 mutations constructed at 38 positions in the C-terminal domain of TraR, nine mutations caused an increase in accumulation between 1.25 and 2 fold (White and Winans, 2005). In a similar study of the N-terminal domain 117 mutations were made at 103 positions, none of which over-accumulated significantly (Costa *et al.*, 2009). In this study we tested six of these mutants and found that all of them have a longer half life than wild type TraR. Together, this data provides additional evidence that the C-terminal domain of TraR targets the protein for proteolysis.

Residue Leu233 was identified as the second possible protease recognition site. Deletion of the C-terminal residue (Ile234) caused a strong defect in function (Luo *et al.*, 2003), probably because the C-terminal carboxyl group forms an ionic bond with Arg230 of the opposite subunit (Vannini *et al.*, 2002; Zhang *et al.*, 2002). Leu233 and Ile234 were each altered to alanine and valine, which caused a loss in positive control, DNA binding, and accumulation (White and Winans, 2005). It would be interesting to alter these residues to hydrophilic ones in an otherwise wild type protein. We have tested the addition of two extra TraR residues Ser235 and Glu236 at the end of TraRCTD and showed that the presence of these two amino acids increased the stability of the protein. This is further evidence that Leu233 and Ile234 decrease TraR stability. Lys221-Lys232 of each subunit form an alpha helix that dimerizes with its counterpart of the opposite subunit. However, Leu233 and Ile234 are not part of this helix. Leu233 is probably highly exposed to solvent, while Ile234 is somewhat more sequestered. It is tempting to speculate that these strongly hydrophobic residues may have evolved precisely to enhance protein turnover. This would be analogous to the two alanine residues at the C-terminus of the *C. crescentus* CtrA protein, which causes the protein to be degraded by the ClpXP protease (Jenal and Fuchs, 1998; Jenal, 2009). In our study, we show that ClpXP, Lon and HSLVU proteases are involved in the degradation of TraRCTD. Apo-TraR was previously reported to be more stable in the double mutant *clpPX, lon* (Zhu and Winans, 2001). Lon and HSLVU were found to be involved in the

degradation of SulA, a cell division inhibitor, in *E. coli* (Kanemori *et al.*, 1999; Seong *et al.*, 1999). HslVU and Lon recognition sites were found to be distinct, but both lie in the center and in the C-terminal domain of SulA. These regions of SulA are involved in molecular interactions with other proteins and are also important for the function of the protein as an inhibitor of cell division (Nishii and Takahashi, 2003).

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[3]CHAPTER 4

The Antiactivator Protein TraM Targets the Quorum Sensing Transcription Activator TraR to Proteolysis

4.1. Summary

The tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* encodes two proteins, TraI and TraR that enable it to communicate via diffusible acyl-homoserine lactone (AHL) pheromones. TraI synthesizes the pheromone, while TraR is a pheromone-dependent transcription factor. TraR activity is inhibited by the TraM protein, by forming a complex containing two copies of each protein. These complexes are unable to bind *tra* box DNA sequences. The fate of TraR-TraM complexes in the cell has not been investigated. In this study, we show that TraM sharply decreased the accumulation of TraR in whole cells, indicating that TraM targets TraR for proteolysis. In contrast, the TraM component of these complexes is not proteolyzed. Point mutations in either protein that block antiactivation also block TraR proteolysis.

[3]Chapter 4 is a manuscript prepared for submission. Costa, E.D., and Winans, S.C. The antiactivator protein TraM targets the quorum sensing transcription activator TraR to proteolysis.

4.2. Introduction

Many types of bacteria can monitor their population densities and coordinate a broad range of physiologies by exchanging chemical signals (Eberl and Riedel, 2011; Galloway *et al.*, 2011; Ng and Bassler, 2009). These communication systems require one or more proteins to synthesize and export the signal and one or more proteins to detect it. Many proteobacteria synthesize acyl-homoserine lactone (AHL) signals via a protein that resembles LuxI of *Vibrio fischeri*, and detects AHLs via transcription factors that resemble LuxR (Pappas *et al.*, 2004; Stevens *et al.*, 2011).

One of the best studied examples of this family of signaling systems is from in the plant pathogen *Agrobacterium tumefaciens*, which is responsible for the crown gall disease (White and Winans, 2007a). The signal molecule is 3-oxo-N-octanoyl homoserine lactone (OOHL), which is produced by the AHL synthase protein TraI, and detected by the AHL receptor TraR, which forms active homodimers. The dimerized TraR binds to specific sequences of DNA called *tra* boxes and activates transcription of the *rep* and *tra* operons, which direct vegetative replication and conjugative transfer, respectively (Fuqua *et al.*, 1996; Pappas and Winans, 2003; Qin *et al.*, 2000; White and Winans, 2007b). TraR is composed of two domains connected by a flexible linker. The amino terminal domain (NTD) contains the OOHL binding site and alpha-helix 9 which mediates dimerization. The carboxyl terminal domain (CTD) contains

a four helix bundle that binds to the *tra* box DNA. The CTD also contributes to protein dimerization (Zhang *et al.*, 2002).

TraR requires OOHL for folding into a protease resistant form, and in the absence of OOHL, it is degraded rapidly by the Clp and Lon proteases, with a half life of approximately 2 minutes, (Zhu and Winans, 2001). TraR-OOHL complexes are inhibited by two antiactivator proteins, TrlR and TraM. TrlR is a truncated form of TraR, which resembles TraR-NTD, and can form heterodimers with TraR that contain just one CTD and are therefore unable to bind DNA (Chai *et al.*, 2001; Oger *et al.*, 1998; Zhu and Winans, 1998). TraM is a small protein that forms a 1:1 complex with TraR. TraR activates the transcription of the *traM* gene, creating a negative feedback loop (Fuqua *et al.*, 1995; Hwang *et al.*, 1995).

The crystal structure of TraM revealed that TraM is a dimer with each subunit consisting of two long antiparallel helices. The complex was also characterized *in vitro* demonstrating that TraM binds TraR with a 2:2 stoichiometry (Chen *et al.*, 2004; Vannini *et al.*, 2004). An orthologous complex containing TraR and TraM from *Rhizobium* sp. NGR234 was visualized by X-ray crystallography (Chen *et al.*, 2007). In this co-crystal, a dimer of TraM contacts a dimer of TraR, and each TraM subunit contacts both domains of TraR, more extensively the CTD, forcing the two CTDs apart, so that they cannot bind to a *tra* box (Chen *et al.*, 2007).

Little is known about the fate of TraM-TraR complexes after binding. In this study we show TraR is degraded in the presence of TraM. When TraR

mutants that interfere with the complex formation were used, the accumulation of TraR in the presence of TraM increased. We also demonstrate that the concentration of TraM in the cells is independent of the concentration of TraR. From this, we can conclude that TraM targets TraR to proteolysis and that it itself is spared from that fate.

4.3. Materials and Methods

Bacterial strains, plasmids, and oligonucleotides

Bacterial strains and plasmids used in this study are listed in Table 4.1. *A. tumefaciens* strains were cultured in AT minimal medium at 28°C (Tempé *et al.*, 1977). *Escherichia coli* strains were cultured in LB medium. Synthetic OOHL was provided by A. Eberhard (Cornell University). Antibiotics were added to maintain plasmids at the following concentrations: 100 µg/mL spectinomycin, and 15 µg/mL gentamycin for *E. coli*; and 200 µg/mL spectinomycin and 200 µg/mL gentamycin for *A. tumefaciens*. The oligonucleotides used in this study are listed in Table 4.2 and were obtained from Integrated DNA Technologies (Coralville, Iowa). Restriction enzymes were obtained from New England Biolabs.

Table 4.1. Bacterial strains and plasmids used in this study

Strains	Relevant genotype	Reference
NTL4	*A. tumefaciens* derivative of strain C58, lacking Ti plasmid	(Luo *et al.*, 2001)
DH5α	*E. coli* , α-complementation	Stratagene
Plasmids		
pPZP200	Broad-host-range cloning vector, SpR	(Hajdukiewicz *et al.*, 1994)
pYC335	*traR* cloned into EcoRI and BamHI sites of pPZP201	(Chai and Winans, 2004)
pSRKGent	Broad-host-range cloning vector, replication and partitioning genes of pBBR1, GentR, lacIq	(Khan *et al.*, 2008)
pEC500	TetR and Ptet cloned into pPZP200	This Study
pEC501	*traR* cloned under control of PTet from Tn10	This Study
pEC505	*traM* cloned into NdeI and BamHI sites of pSRKGent	This Study
pEC508	Flag-tag-*traM* cloned into NdeI and BamHI sites of pSRKGent	This Study
pEC526	182F substitution of TraR in pEC501	This Study
pEC527	195T substitution of TraR in pEC501	This Study
pEC528	195V substitution of TraR in pEC501	This Study

DNA manipulations

Recombinant DNA techniques were performed using standard procedures (Sambrook and Russell, 2001). Plasmid DNA was isolated from *E. coli* with QIAprep spin miniprep kits (Qiagen) for DNA sequence analysis. DNA sequences of constructs that were obtained by PCR were verified using automated DNA sequencing (Cornell Biotechnology Resource Center) and analyzed using the LaserGene program (DNASTAR). Plasmids were introduced into *E. coli* by transformation (Sambrook and Russell, 2001) and

into *A. tumefaciens* by electroporation (Cangelosi *et al.*, 1991). *E. coli* strain DH5α was used for all plasmid constructions.

Plasmid construction

The construction of the plasmid pEC500 containing the Ptet and *tetR* was performed using transposon Tn10 as a template. A 783 bp fragment of Tn10 containing *tetR*, Ptet, operator regions, and 18 bp of *tetA* (three stop codons were added at the end of the tetA fragment) was amplified by PCR using the oligonucleotidesTn10 F and Tn10 R listed in Table 4.2. The resulting fragment was digested with *EcoRI* and *SacI* and cloned into the same restriction sites of pPZP200. The resulting plasmid is pEC500.

To construct the plasmid expressing TraR, the *traR* gene was PCR amplified with the use of plasmid pYC335 (Chai and Winans, 2004) as a template and the oligonucleotides (TraR F and TraR R) listed in Table 4.2. The resulting DNA fragment was digested with *Kpn*I and *Bam*HI and cloned into pEC500 digested with the same enzymes, resulting in plasmid pEC501.

The plasmid expressing TraM was constructed by amplifying *traM* from the genome of *A. tumefaciens* strain R10 using the oligonucleotides TraM F and TraM R listed in Table 4.2. The resulting PCR fragment was cloned into NdeI and BamHI sites of pSRKGent (Khan *et al.*, 2008) resulting in pEC505. To construct Flag-TraM, the *traM* gene was PCR amplified using the oligonucleotides (Flag-tag-TraM F and TraM R) listed in Table 4.2. The resulting fragment was digested using NdeI and BamHI and cloned into the same sites of pSRKGent (Khan *et al.*, 2008) resulting in plamid pEC508.

Table 4.2. Oligonucleotides used in this study.

Oligonucleotides	Sequences
Tn10 F	5'-GCTGAATTCTAACTCGACATCTTGGTTAC-3'
Tn10 R	5'-GCTGAGCTCTTATCACTACTTTGTCGAACTATTCAT-3'
TraR F	5'-GCTGGTACCTAGGAGGTATGGAATGCAGCAC-3'
TraR R	5'-GCTGGATCCACTTCGAACTCTCAGATGAG-3'
TraM F	5'-GCTCATATGATGGAACTGGAAGATGC-3'
TraM R	5'-GCTGGATCCTCAGTTGACGACCACCT-3'
Flag-tag-TraM F	5'-GCTCATATGGATTACAAGGATGATGATGATAAGGAATCGGAAGAT GCAACATTG-3'
L182F F	5'-GCCACCTATTTCAGATGGAT-3'
L182F R	5'-ATCCATCTGAAATAGGTGGC-3'
A195T F	5'-GAGGAGATCACCGACGTCGA-3'
A195T R	5'-TCGACGTCGGTGATCTCCTC-3'
A195V F	5'-GAGGAGATCGTCGACGTCGA-3'
A195V R	5'-TCGACGTCGACGATCTCCTC-3'

Site-directed mutagenesis

Site-directed mutagenesis of TraR was performed using synthetic overlap extension PCR (Sambrook and Russell, 2001). A 747 bp fragment of plasmid pEC501 was amplified using *Taq* polymerase High Fidelity (Invitrogen). The restriction sites for *KpnI* and *BamHI* were used to introduce mutated DNA fragments into the wild type gene.

Immunodetection of TraR

The abundance of each TraR and TraM was determined in *A. tumefaciens* in the following way. A portion of each culture was centrifuged and the cell pellets were resuspended in 5% of their original volume in 1x

cracking buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, 0.02% bromophenol blue). Cells were disrupted by boiling for 5 min, cooling to -80°C and boiling for another 5 min. A fraction of each sample was size-fractionated using 12% SDS polyacrylamide gels, and electroblotted onto nitrocellulose membranes (BIORAD). The membranes were blocked using TBS (20 mM Tris pH 7.9, 500 mM NaCl, 0.05% Tween 20) supplemented with 5% skim milk. TraR was immunodetected in TBS with pre-adsorbed polyclonal anti-TraR rabbit antiserum (Chai and Winans, 2004) and goat anti-rabbit IgG conjugated with alkaline phosphatase (BIORAD) was used as the secondary antibody. TraM was immunodetected in TBS with monoclonal anti-Flag M2 antibody produced (Sigma-Aldrich). Goat anti-mouse IgG conjugated with alkaline phosphatase (Jackson immunoResearch Laboratories) was used as the secondary antibody. The membranes were stained with BCIP (5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt) and NBT (p-nitro blue tetrazolium chloride) (BIORAD). Westerns were performed with fresh cell lysates for each strain at least three times. Data were analyzed using ImageJ (http://rsb.info.nih.gov/ij/) (Rasband, 2004), and normalized against cross-reacting material in each lane.

OOHL sequestration assay by TraR in whole cells

A. tumefaciens strain NTL4 containing both pEC501 and pEC508 plasmids was used for OOHL sequestration assays as previously described (Chai et al., 2001).

4.4. Results

TraM enhances TraR proteolysis

It was previously found that binding of TraM to TraR prevents TraR from binding DNA (Chen *et al.*, 2007). However, the ultimate fate of the TraR-TraM protein complexes was not known. To determine whether either of these proteins was degraded, we constructed two plasmids: pEC501, which express TraR constitutively from a P*tet* promoter, and pEC508, which expresses P*lac*-*traM* fusion, and also contains *lacI*^{Q}, so that expression of the fusion can be controlled using IPTG. The plasmid pEC501 has a P*tet*-TraR and encodes the repressor proteinTetR. The induction of the TraR expression should be controlled by the addition of anhydrotetracycline, but for unknown reasons, the expression of the P*tet-traR* in pEC501 is constitutive. Even though expression of the *traR* gene was constitutive, the abundance of TraR protein was controlled using OOHL, as apo-TraR is degraded almost immediately. These plasmids were introduced into *A. tumefaciens* strain NTL4, which lacks the Ti plasmid, cultured to late log phase in the presence of varying concentrations of OOHL and IPTG, and analyzed for TraR and TraM contents by western immunoblotting.

In experiments to detect TraR degradation, we used a low level of OOHL (0.1 nM) and varied concentrations of IPTG. As expected, the abundance of TraM was highly responsive to IPTG concentration. TraM was not detected after growth in the presence of 0, 40, or 80 µM IPTG, and expression was saturated by growth in 1280 µM (Fig 4.1 and Table 4.3).

Significantly, the abundance of TraM and TraR were inversely correlated. As high levels of TraM, TraR was undetectable. In control experiments, TraR did not accumulate in the absence of OOHL, and the addition of IPTG to cells lacking *traM* did not alter TraR abundance (data not shown).

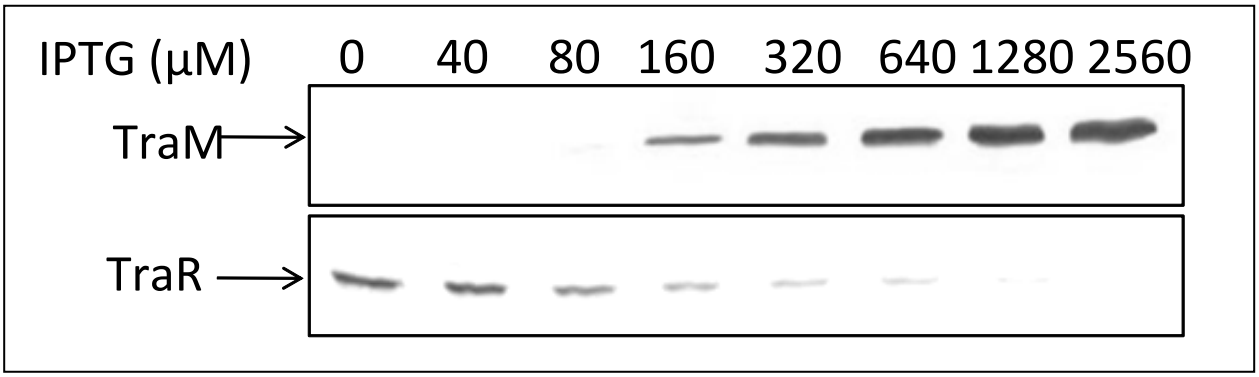

Figure 4.1. TraR degradation with increasing amounts of TraM . Western immunoblots of NTL4(pEC501)(pEC508) cells cultured in the presence of 0.1 nM OOHL with different concentrations of IPTG (indicated in the top of the figure).

We did similar experiments using higher concentrations of OOHL, which enhanced the abundance of TraR. In the presence of 1 nM OOHL, ITPG caused a small decrease in TraR abundance, while in the presence of 10 nM or 100 nM OOHL, IPTG had no effect (Table 4.3). This provides suggestive evidence that TraM acts stoichiometrically rather than catalytically to degrade TraR.

Table 4.3. TraR accumulation using different concentrations of OOHL and IPTG. The accumulation was assessed by Western immunoblots.

	TraR Abundance (% of TraR Abundance without IPTG)*							
OOHL (nM)	IPTG (μM)							
	0	40	80	160	320	640	1280	2560
0.1	100	98	71	26	14	11	< 10	< 10
1	100	96	102	98	87	72	69	63
10	100	NT	NT	NT	NT	106	107	101
100	100	NT	NT	NT	NT	104	104	107

* Accumulation was determined considering the protein and a load control (cross reacting material). NT: not tested

A second method was used to confirm and quantify the accumulation of TraR in the presence or absence of TraM. We measured the sequestration OOHL in the absence of IPTG or in the presence of 1 mM IPTG. We cultured cells expressing TraR and TraM or only expressing TraR in the presence of 0.1 nM of OOHL , then washed the cells five times, extracted the OOHL using an organic solvent, and bioassayed the extracted OOHL. The strain expressing both TraR and TraM sequestered 90% less OOHL than the cell expressing only TraR (Fig.4.2). The sequestration of OOHL by the cells that do not express TraR was less than 1% (Fig 4.2). These data confirm the western immunoblots described above, and indicate that TraM causes the destruction of TraR.

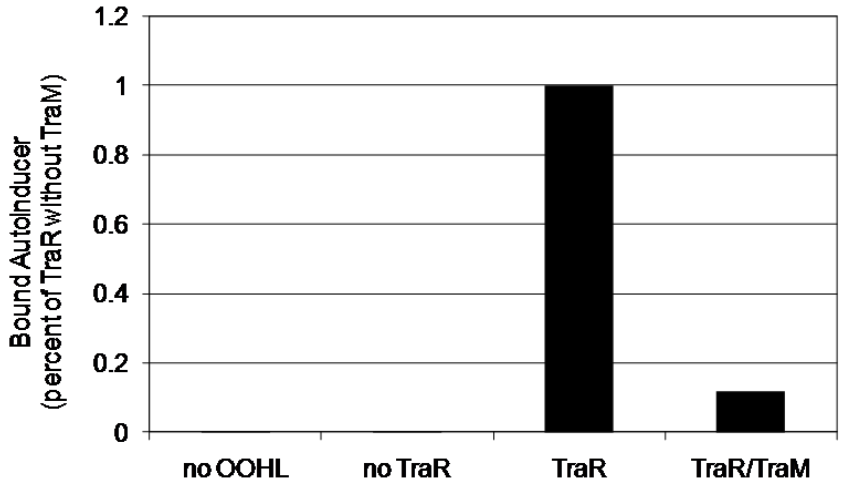

Figure 4.2. TraR sequesters less OOHL in the presence of TraM than in its absence. OOHL sequestration assays. The assay was performed using 0.1 nM OOHL and 1 mM IPTG.

Accumulation of TraR in the presence of TraM increases when the protein binding between TraR and TraM is impeded

Previous studies have shown that particular amino acids in the carboxyl terminal domain of TraR are involved in the interaction with TraM (Chen *et al.*, 2007; Luo *et al.*, 2000; Qin *et al.*, 2007). TraR mutations L182F, A195T, and A195V interfere with antiactivation by TraM (Luo *et al.*, 2000; Qin *et al.*, 2007). The abundance of TraR proteins containing these mutations was measured in the absence and presence of TraM. As expected, TraM was not detected in the absence of IPTG, but could be detected when 1 mM of IPTG was used (Fig. 4.3A). Also as expected, the abundance of wild type TraR was severely decreased by IPTG (compare Fig. 4.3B. lanes 1 and 2). In contrast, TraR(L182F), TraR(A195T) and TraR(A195V) were still easily detected in the presence of IPTG (Fig 4.3B compare lanes 2, 4, 6, and 8). The intensities of

these bands are quantified in Table 4.4. The accumulation of TraR was not completely restored with the mutations (Fig 4.3B and Table 4.4). This result can be explained by the fact that the single mutations in TraR do not disrupt completely the interaction between TraM and TraR. In fact, these mutants were found to interfere with TraM/TraR binding, but they seem not to be directly involved in the interaction between the proteins (Chen *et al.*, 2007; Qin *et al.*, 2007). The possible role of these amino acids in the interaction between the proteins will be further discussed below.

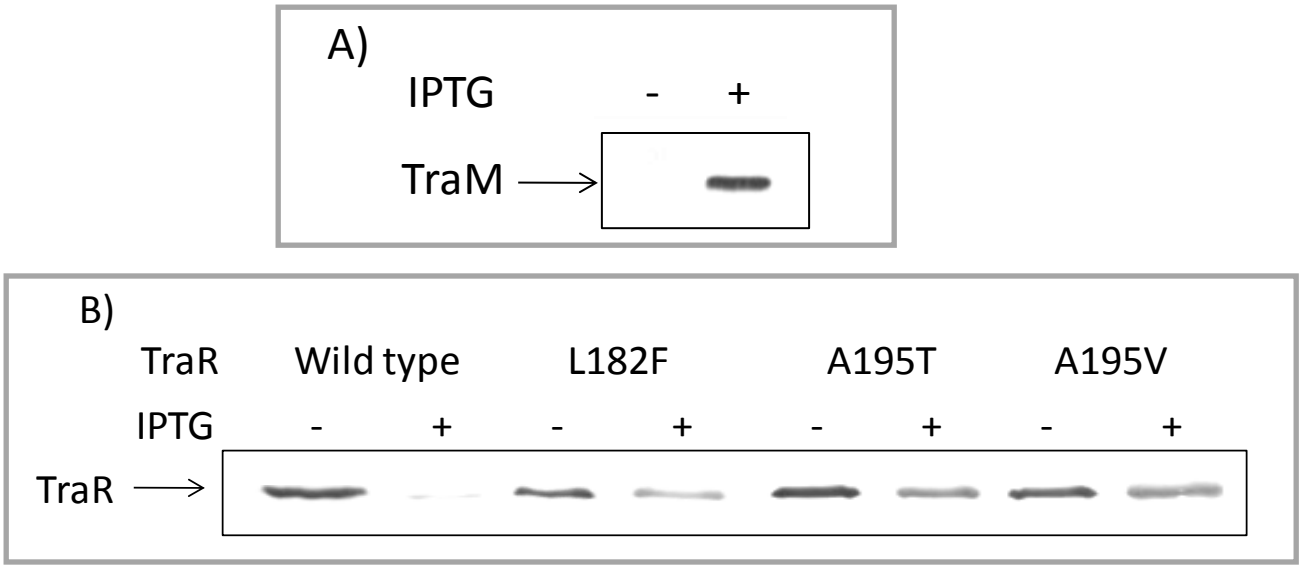

Figure 4.3. Mutations in TraR that interfere with the interaction between this protein and its antiactivator are more stable than wild type TraR in the presence of TraM. (A) Accumulation of TraM without the addition of IPTG (-) and with the addition of 1 mM of IPTG (+). (B) Western immunoblots of TraR in the presence or the absence of 1 mM IPTG.

Table 4.4. Accumulation of TraR wild type and mutants, L182F, A195T and A195V, in the presence or absence of TraM.

	Accumulation*	
TraR	No IPTG (no TraM)	1mM IPTG (TraM)
Wild type	100	< 10
L182F	100	44
A195T	100	56
A195V	100	67

*the accumulation was calculated considering each allele of TraR without the addition of IPTG as 100% of accumulation. The intensity of the bands was normalized against a load control.

TraM levels do not decrease with increasing amounts of active TraR

Data in the previous section suggest that TraM targets TraR for proteolysis. We wanted to determine whether the converse is also true, that is, whether TraR can target TraM for degradation. To test this, we cultured the same strain in the presence of 160 µM IPTG, and 0, 1, 10, 100, or 1000 nM OOHL. This IPTG concentration causes a very low but detectable accumulation of TraM, while the varied OOHL concentrations result in different concentrations of TraR.

As expected, increasing amounts of OOHL caused increasing abundance of TraR (Figure 4.4). However, the abundance of TraM was constant in all cultures. These results indicate that TraM is not degraded after interaction with TraR, and it may work as an adaptor that presents TraR to proteases.

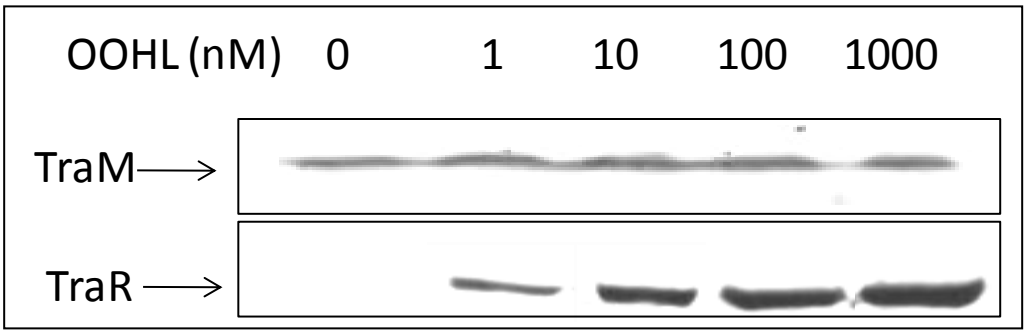

Figure 4.4. The presence of TraR does not change TraM stability. Western immunoblots of TraM and TraR in the presence of 160 µM of IPTG and increasing concentrations of OOHL (indicated at the top of the figure).

The *traM* leader sequence contains a 300 nucleotide highly conserved sequence.

It is far from clear what benefit would be obtained from the antiactivation of TraR by TraM. If TraM is always induced by TraR and always opposes TraR function, why not simply make less TraR? It seemed at least plausible that antiactivation might be responsive to an environmental stimulus, which could act either at the transcriptional level, post-transcriptional level, or at the protein level. We noticed that the *traM* leader sequence is unusually long, and that it is strongly conserved, even more so than the *traM* coding sequences (Figure 4.5). We used BLASTN to search for similar sequences in published DNA sequence databases, and chose ten such sequences for further analysis. All *traM* orthlogs have similar long upstream sequences, although it is not clear whether they are transcribed (Figure 4.6). Additional similar sequences were found upstream of members of the XRE family of

transcription factors (Figure 4.6). All homologous sequences were found in members of the alphaproteobacteria. The MFOLD algorithm was used to look for possible secondary structures of the *traM* leader of pTiR10. A predicted structure with high stability was detected (Figure 4.7).

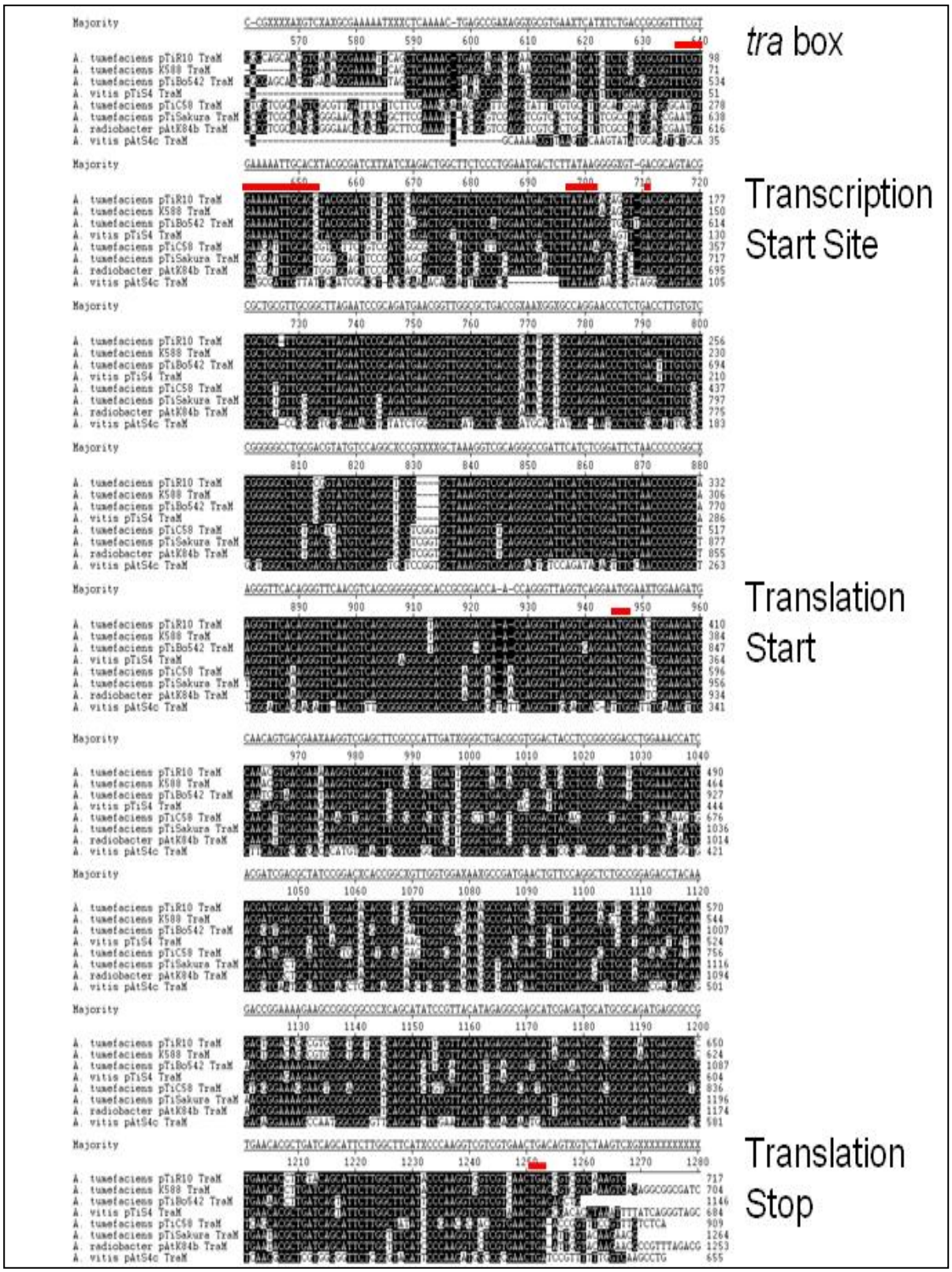

Figure 4.5. Alignment of eight *traM* genes with upstream regions. As seen above, the leader sequences are more strongly conserved than the *traM* reading frames.

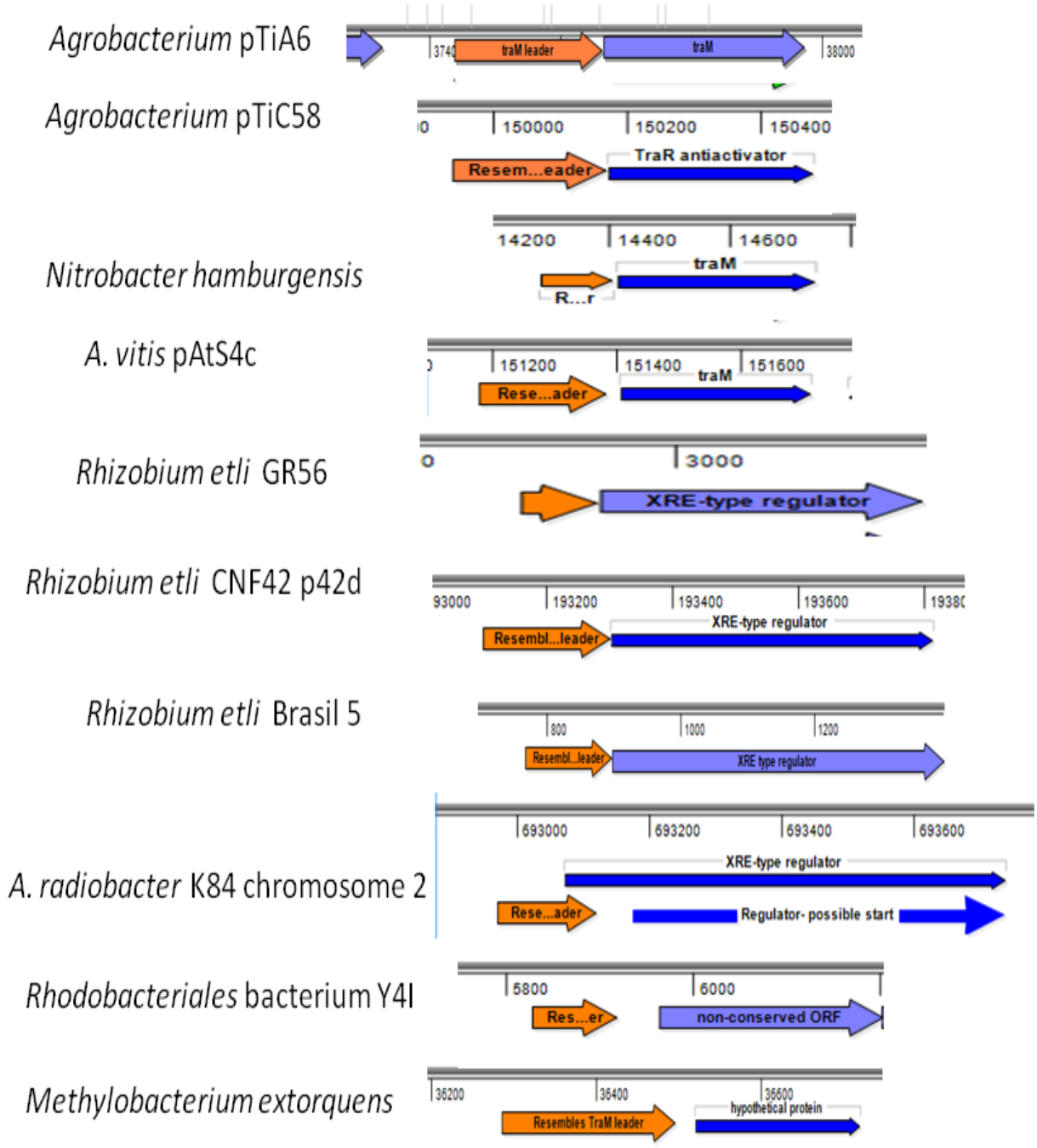

Figure 4.6. The top four lines show conservation of the leader and *traM* gene in four plasmids. The four middle lines show examples of sequences that resemble the *traM* leader and lie upstream of members of the Xre family of transcription regulators. The bottom two lines show homologs of the *traM* leader that lie upstream of genes that do not resemble *traM*.

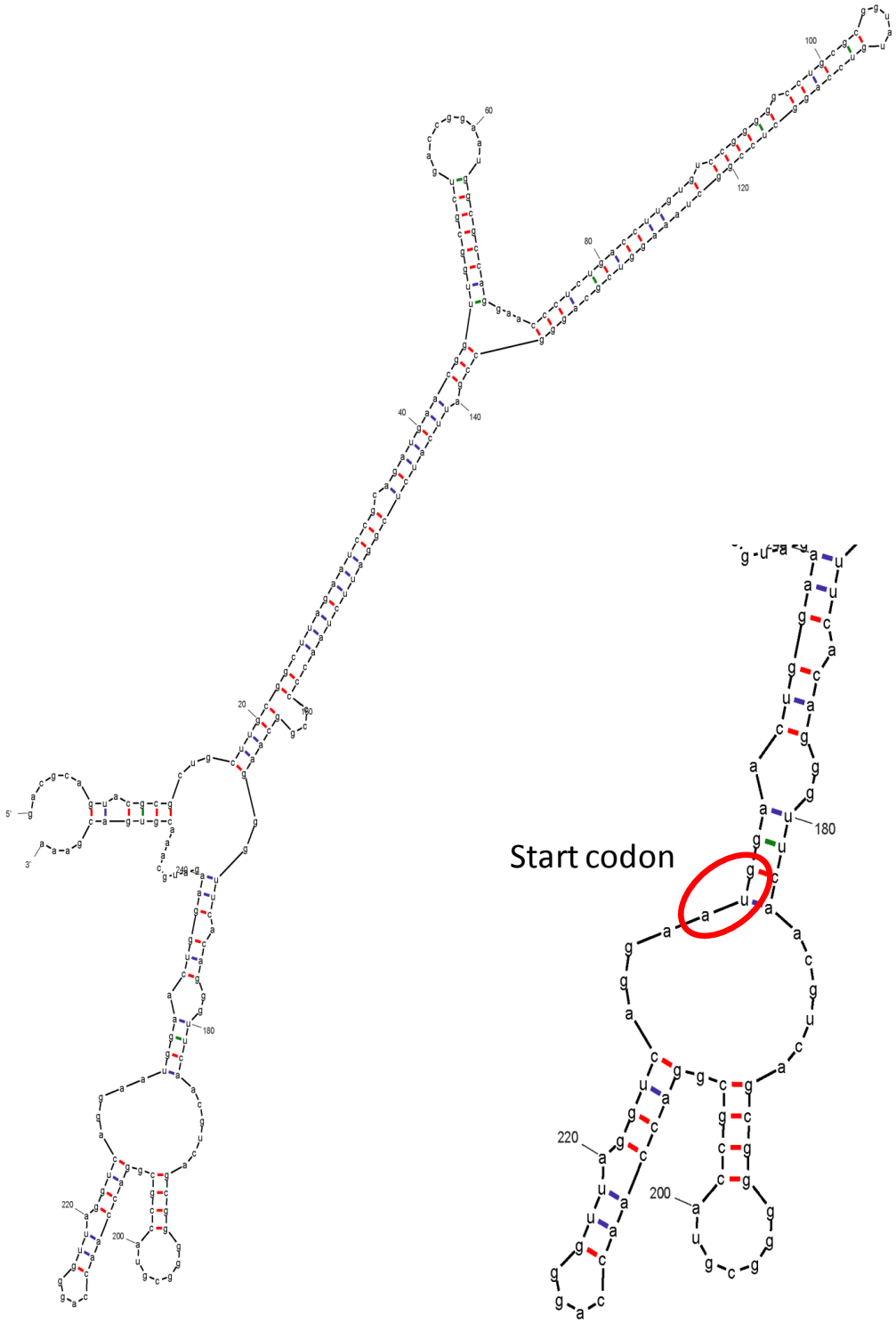

Figure 4.7. Predicted secondary structure of the *traM* leader of pTiR10 (dG = -105.39).

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4.5. Discussion

In this study we sought to determine the fate of complexes containing TraR and TraM. Our first hyphotesis was that TraR was degraded after being inactivated by TraM. We demonstrated that, as expected, TraR levels decreased in the presence of TraM (Fig. 4.1). To confirm that the interaction between TraR and TraM is involved in the observed degradation of TraR in our Western immunoblots, we constructed mutants in TraR (L182F, A195V and A195T) that were shown previously to be resistant to TraM inhibition (Luo *et al.*, 2000). The protein products of three TraR mutants were detected in higher abundance than the wild type protein in the presence of TraM by Western analysis. The co-crystal structure of TraM and TraR from *Rhizobium* sp. NGR234 (Chen *et al.*, 2007) showed that helices α10 and α11 of TraR have most of the amino acids involved in the interaction with TraM. Residue L182 is present in α10 and residue A195 in α11. L182 was shown to project its side chain into the groove formed between the two TraM alpha helices (Chen *et al.*, 2007). When we used these mutations in our study, they did not restore the accumulation of TraR to 100%. This result is in agreement with the fact that these mutations do not completely eliminate the interaction between TraR and TraM as described previously (Luo *et al.*, 2000; Qin *et al.*, 2007).

The finding that the interaction between TraM and TraR causes a decrease in TraR abundance led us to test the accumulation of TraM in the presence of increasing levels of TraR. The levels of TraM did not change

when different concentrations of active TraR were used (Fig. 4.4). If any degradation had occurred, it should have been detectable, as we expressed TraM at low levels and expressed TraR at varying levels, including levels that were much higher than TraM (Table 4.3). Our results indicate that TraM targets TraR for proteolysis, and that TraM is not degraded in this process, suggesting that it could act catalytically in TraR degradation.

A possible mechanism for TraR degradation is based on the fact that TraM causes an allosteric conformational change in TraR. This conformational change might expose amino acids that are recognized by proteases as degradation signals leading TraR to proteolysis. However, TraM would have to be released from the complex before the degradation of TraR because our results suggest that TraM is not degraded with TraR.

Another possibility is that TraM works as an adaptor protein which mediates the interaction between TraR and proteases, enhancing proteolysis of this transcription activator.

In some cases, proteins are recognized directly by cellular proteases, while in other cases, substrate recognition can be mediated by proteins called adaptors. Adaptor proteins are normally small and they can deliver specific substrates to the proteases or they can also prevent degradation of specific substrates (Dougan *et al.*, 2002; Roman-Hernandez *et al.*, 2009). Some adaptor proteins that have been described in bacteria are: ClpS, RssB, and SspB in *E. coli* (Flynn *et al.*, 2004; Zhou *et al.*, 2001), and MecA and Ypbh in *B. subtilis* (Nakano et al., 2002; Turgay et al., 1997). Some adaptors, such as

SspB, can regulate the degradation of different classes of proteins (Flynn *et al.*, 2004). Our results suggest that it is possible that TraM works as an adaptor protein which not only inactivates TraR, but also delivers it for degradation. TraM is important to prevent unnecessary expression of the *tra* genes.

The adaptive benefit of TraM is far from clear. One possibility is that TraM is optimally active only in response to some environmental condition. If so, this stimulus could act at the level of *traM* transcription or translation or TraM function. It may be of some significance that the *traM* transcript has a leader of approximately 300 nucleotides that is highly conserved, found upstream of a number of different genes, and that has a very strong predicted secondary structure. The most obvious paradigm for such a sequence would be a riboswitch. If this leader allowed transcription or translation of TraM only under certain conditions, those conditions would indirectly impact the abundance of TraR and the transcription of TraR-regulated genes. We note that there appears to be a riboswitch that impacts the expression of *traR* (A.L. Flores Mireles, unpublished data) that is responsive to cytoplasmic methionine and cysteine.

4.6. References

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CHAPTER 5

Conclusions and Future Work Directions

In this dissertation I have described the possible mechanism of transcription activation by the quorum sensing transcription activator protein TraR. I also showed that the carboxyl terminal domain of this protein is recognized for degradation and that the antiactivator protein, TraM, targets TraR to proteolysis.

In chapter 2, the amino acids in the amino terminal domain of TraR that are important for the interaction with the RNA polymerase were identified. The patches of amino acids in TraR and their possible contacts with the alpha subunit or with another subunit of the RNA polymerase were discussed. In further studies, it would be interesting to identify which subunits of the RNA polymerase are participating in the interaction with each patch of TraR at class I and II promoters. Stop codon mutants were constructed at different positions on TraR surrounding the patches of amino acids found. The idea of this is to add unnatural amino acids to these stop codons by using engineered plasmids which encode tRNA synthetase and tRNA capable of incorporation of these unnatural aminoacids at the stop codons of TraR. One example of these plasmids is pEVOL which was obtained by our lab from the laboratory of Dr. Peter G. Schultz (The Scripps Research Institute). The use of unnatural amino acids, such as p-Benzoylphenylalanine, would allow crosslinking between

TraR and different subunits of the RNA polymerase. The position where the crosslinks occur in the RNA polymerase could be mapped by mass spectroscopy. Another experiment that could be done is the construction of point mutations in the alpha subunit and the region 4 of sigma of the RNA polymerase. Techniques such as in vitro transcription, surface plasmon resonance and fluorescence anisotropy could be performed with the mutants RNA polymerase and TraR to determine which amino acids of the RNA polymerase are involved in the interactions with this transcription factor.

In chapter 3, it was shown that amino acids in the carboxyl terminal domains of TraR target the protein to proteolysis. It is fascinating that amino acids involved in the contacts between the TraR and DNA or TraR and RNA polymerase destabilize the protein. Amino acids buried in the dimer TraR and surface exposed in the monomeric protein are also targets for degradation. These results combined indicate that if TraR is monomeric or dimeric, but not bound to either DNA or RNA polymerase, it is automatically targeted for proteolysis.

In Chapter 4, it was found that TraR is degraded after interaction with TraM, and that TraM is not degraded in this process, suggesting that TraM might work as an adaptor protein, increasing the rate of degradation of TraR. Another possibility is that the conformational change of TraR by TraM exposes amino acids that are recognized by proteases, resulting in proteolysis. Adaptor proteins increase specificity of proteases to substrate and accelerate their rate of degradation. The adaptor proteins bind to the ATPase domain of the

protease complexes and deliver the substrate to the processing pore site of the ATPase. It would be interesting to check if TraM interacts with ClpA, ClpX, Lon or HslU from *A. tumefaciens.* The interactions could be tested by bacterial two hybrid system or by crosslinking. If the interactions are verified, the amino acids involved in the interaction could be mapped and the mechanism of interaction and delivery of TraR to the proteases described. Adaptors are known to be well regulated by the cells. In Chapter 4 we also discussed the presence of a 300 bp untranslated leader sequence upstream of *traM* which was predicted to form a secondary structure. It would also be interesting to test the function of the leader sequence in the regulation of TraM. Resections of the leader sequence could be done and fused to LacZ. This leader sequence might be a riboswitch which is regulated by some small molecule. Further studies investigating the function of the leader sequence upstream of *traM* and the role of this protein as an adaptor between TraR and proteases could bring new insights about the regulation of TraR.

[4]APPENDIX

Saturation Mutagenesis of a CepR Binding Site as a Means to Identify New Quorum-regulated Promoters in *Burkholderia cenocepacia*

A.1. Summary

Burkholderia cenocepacia is an opportunistic pathogen of humans that encodes two genes that resemble the acylhomoserine lactone synthase gene *luxI* of *Vibrio fischeri* and three genes that resemble the acylhomoserine lactone receptor gene *luxR*. Of these, CepI synthesizes octanoylhomoserine lactone (OHL), while CepR is an OHL-dependent transcription factor. In the current study we developed a strategy to identify genes that are directly regulated by CepR. Yuping Wei and I systematically altered a CepR binding site (*cep* box) upstream of a target promoter to identify nucleotides that are essential for CepR activity *in vivo* and *in vitro* for CepR binding. Yuping Wei constructed 34 self-complementary oligonucleotides containing altered *cep* boxes, and measured binding affinity for each. These experiments allowed us to identify a consensus CepR binding site. Several hundred similar sequences were identified by David Schneider and Stephen Winans, some of which were adjacent to probable promoters. Ana Flores-Mireles fused several such

[4] Wei, Y., Ryan, G.T., Flores-Mireles, A.L., Costa, E.D., Schneider, D.J., and Winans, S.C. (2011) Saturation mutagenesis of a CepR binding site as a mean to identify new quorum-regulated promoters in *Burkholderia cenocepacia. Mol Microbiol* **79**(3): 616-632.

promoters to a reporter gene with and without intact *cep* boxes. This allowed us to identify four new regulated promoters that were induced by OHL, and that required a *cep* box for induction. CepR-dependent, OHL-dependent expression of all four promoters was reconstituted in *E. coli* by Gina Ryan who showed that purified CepR bound to each of these sites in electrophoretic mobility shift assays.

A.2. Introduction

The genus *Burkholderia* encompasses a fascinating collection of diverse β-proteobacteria (Coenye and Vandamme, 2003). This genus includes over 50 species, some of which are potentially useful in bioremediation, while other members are capable of forming nitrogen-fixing root nodules with legumes (Bontemps *et al.*, 2010; Chen *et al.*, 2003). Some members protect host plants against fungal pathogens, while others are themselves pathogenic against plants, animals, and humans (Coenye and Vandamme, 2003; Jones and Webb, 2003). Seventeen pathogenic species are members of the *Burkholderia cepacia* complex, or BCC (Vandamme *et al.*, 1997; Vanlaere *et al.*, 2008; Vanlaere *et al.*, 2009), two of which are described by the Center for Disease Control as category B select agents (Godoy *et al.*, 2003).

B. cenocepacia, previously known as *B. cepacia* genomovar III (Vandamme *et al.*, 2003), is recognized as an opportunistic pathogen of humans and is a particular threat to cystic fibrosis (CF) patients (Mahenthiralingam *et al.*, 2005; Vandamme *et al.*, 1997). Colonization of the

CF lung by *B. cenocepacia* (Vandamme *et al.*, 2003) tends to occur in patients already infected with *Pseudomonas aeruginosa*, another opportunistic pathogen of the CF lung (Jones and Webb, 2003; Vandamme *et al.*, 1997). An infection caused by both organisms can result in serious clinical complications. *B. cenocepacia* strains are resistant to most antibiotics, making them virtually impossible to eradicate (Nzula *et al.*, 2002). Infections with *B. cenocepacia* may have variable clinical outcomes ranging from asymptomatic carriage to a sudden fatal deterioration in lung function (Mahenthiralingam *et al.*, 2005).

Four strains of *B. cenocepacia* have been sequenced in their entirety, one of which is described in a publication (Holden *et al.*, 2009). The Joint Genome Institute is currently sequencing nine additional strains (http://www.jgi.doe.gov/genome-projects/). All four sequenced isolates have three circular chromosomes that vary in size between 3.9 and 0.88 MB in length. Strains J2315 and HI2424 also have one plasmid, 93 KB and 165 KB in length, respectively.

Many or possibly all *Burkholderia* spp. encode at least one regulatory system that resembles the LuxR and LuxI proteins of *Vibrio fischeri*, where LuxI synthesizes an acylhomoserine lactone (AHL)-type pheromone, also called an autoinducer, and LuxR is an AHL-dependent transcriptional regulator (Choi and Greenberg, 1992; Eberhard *et al.*, 1981; Engebrecht and Silverman, 1984). Regulatory systems of this family are found in countless proteobacteria, where they are thought to allow individual bacteria to coordinate their physiology with their siblings. Collectively, these systems regulate diverse

processes, including pathogenesis, biofilm formation, bacterial conjugation, and the production of antibiotics and other secondary metabolites (Whitehead *et al.*, 2001). In general, target genes are transcribed preferentially at population densities high enough to favor AHL accumulation (Eberhard *et al.*, 1991), a phenomenon referred to as quorum sensing (Fuqua *et al.*, 1994). *B. thailandiensis* has three such systems, one of which is implicated in cell aggregation, while another is required for antibiotic production (Chandler *et al.*, 2009; Duerkop *et al.*, 2009). A plant growth promoting isolate of *B. ambifaria* uses quorum sensing to regulate the production of the anti-fungal compound pyrrolnitrin (Schmidt *et al.*, 2009).

LuxR-type proteins have two domains, an N-terminal pheromone binding domain and a C-terminal DNA binding domain (Pappas *et al.*, 2004). Purified LuxR, TraR of *Agrobacterium tumefaciens*, and LasR of *Pseudomonas aeruginosa*, when complexed with their respective AHLs, bind with high specificity to recognition sequences (referred to as *lux*, *tra,* or *las* boxes, respectively) that are found at target promoters (Schuster *et al.*, 2004; Urbanowski *et al.*, 2004; Zhu and Winans, 1999). LasR is also able to bind to sequences that have no obvious resemblance to canonical *las* boxes. A few members of this family bind DNA only in the *absence* of AHLs (Castang *et al.*, 2006; Cui *et al.*, 2005; Fineran *et al.*, 2005; Minogue *et al.*, 2005; Sjoblom *et al.*, 2006).

Burkholderia cenocepacia J2315 encodes three LuxR homologs and two LuxI homologs (Lewenza *et al.*, 1999; Malott *et al.*, 2005; Malott *et al.*,

2009). Among these, CepR and CepI appear to be well conserved within the BCC (Venturi *et al.*, 2004). CepI synthesizes primarily octanoylhomoserine lactone (OHL), and lower levels of hexanoylhomoserine lactone (HHL) (Aguilar *et al.*, 2003b; Gotschlich *et al.*, 2001; Huber *et al.*, 2001; Lewenza *et al.*, 1999). Null mutations in *cepI* or *cepR* increased the production of the siderophore ornibactin, and decreased the production of secreted lipases and metalloproteases ZmpA and ZmpB (Kooi *et al.*, 2006; Lewenza *et al.*, 1999; Lewenza and Sokol, 2001; Sokol *et al.*, 2003). CepI and CepR are also required for swarming motility and biofilm formation (Huber *et al.*, 2001) and for pathogenicity in several animal models (Kothe *et al.*, 2003; Sokol *et al.*, 2003). *B. cenocepacia* also expresses the CciI and CciR proteins, which are encoded on a genomic island called *cci* (cenocepacia island), that is associated with epidemic strains (Malott *et al.*, 2005). The CepIR and CciIR systems extensively interact, in that CciR negatively regulates *cepI*, while CepR is required for expression of the *cciIR* operon (Malott *et al.*, 2005). Transcriptional profiling studies indicate that CepR and CciR regulate many of the same genes, but do so in opposite ways (O'Grady *et al.*, 2009). *B. cenocepacia* also encodes an orphan LuxR homolog called CepR2, which represses a cluster of genes that may direct production of an antibiotic or other secondary metabolite (Malott *et al.*, 2009).

In addition to transcriptional profiling several other approaches have been used to identify genes whose expression is influenced by CepR and/or OHL. In one study, the proteome of a wild type *B. cenocepacia* was compared

to that of a *cepR* mutant. Fifty-five proteins were found to be differentially expressed in the two strains, approximately 10% of all detected proteins (Riedel *et al.*, 2003). In a second study, fragments of a *B. cepacia* strain were cloned into a promoter trap plasmid and introduced into an *E. coli* strain that expressed CepR (Aguilar *et al.*, 2003a). Twenty-eight promoter fragments were identified as being induced by OHL, and in all cases, induction required CepR. In a third study, a library of *B. cenocepacia* DNA fragments were introduced into a plasmid containing a promoterless *luxCDABE* operon (Subsin *et al.*, 2007). That study identified 58 OHL-inducible promoters and 31 OHL-repressible promoters. Regulation of nine of these genes required CepR, while the others were not tested. Seven OHL-inducible genes were identified by screening a library of *lacZ* fusions (Weingart *et al.*, 2005). Induction of all of these genes required CepR. Purified CepR-OHL complexes bound with high affinity and specificity to specific DNA sequences at two target promoters (Weingart *et al.*, 2005). These binding sites contained a 16-nucleotide imperfect dyad symmetry and were centered approximately 44 nucleotides upstream of the transcription start sites. These two sites are to date the only experimentally confirmed CepR binding sites.

Most of the studies described above do not distinguish whether a target promoter is controlled by CepR directly or indirectly. CepR could regulate a promoter indirectly, for example, by directly regulating an unknown regulatory gene whose product directly regulates that promoter. Alternatively, a CepR

mutation might perturb cellular physiology in such a way that various promoters are affected by secondary effects.

To date, the most comprehensive study attempting to define the optimal CepR binding site was done by Chambers, Sokol, and colleagues (Chambers *et al.*, 2006), who approached this question with an impressive combination of genetics and bioinformatics. Mutagenesis of the known CepR binding site within the *cepI* promoter completely abolished induction (Chambers *et al.*, 2006). The promoters of six genes known to be induced by OHL were used to formulate a consensus CepR binding motif (Chambers *et al.*, 2006). This information was used to test eight additional candidate promoters, six of which were CepR-regulated. Ultimately, ten inducible promoters were used to refine the consensus sequence, and 57 possible CepR binding sites were identified upstream of various genes.

The consensus motif identified in the Chambers study included the sequence CTG-N_{10}-CAG, which has dyad symmetry. However, several other bases in the consensus did not preserve this symmetry, and some of those non-symmetric bases were said to be highly conserved (Chambers *et al.*, 2006). The partial dyad symmetry suggests that CepR binds DNA as dimer and that the two DNA binding domains have rotational symmetry. Although we have no proof of this, structural studies of a related protein support this idea (Vannini *et al.*, 2002; Zhang *et al.*, 2002). Several other LuxR-type proteins are thought to decode dyad symmetrical sequences (Antunes *et al.*, 2008; White and Winans, 2007; Whitehead *et al.*, 2001). In the present study, Gina T. Ryan

tested the 10 putative CepR binding sites described above for the ability to bind purified CepR-OHL complexes. Yuping Wei and I systematically resected and mutated a known CepR binding site, and Ana L. Flores-Mireles used the resulting information to identify four new promoters that are regulated directly by CepR. All four promoters are regulated by CepR *in vivo*, require their binding sites for regulation, and bind with high affinity to CepR-OHL *in vitro.*

A.3. Results

Note that only the results corresponding to the experiments that I did for this study are shown here. For the other figures, see the published manuscript[4].

Systematic mutagenesis of a CepR-dependent promoter

In a previous study, a series of 4 nucleotide alterations were made in the CepR binding site of the *cepI* promoter, and all such mutations that disrupted any part of this binding site blocked promoter activation by CepR (Chambers *et al.*, 2006). In the current study, I have extended those findings by altering single nucleotides within and beyond this site (Fig. A.1). I altered each of these bases to its complement, fused the resulting promoters to *lacZ*, and measured their activity *in vivo*. I used a broad range of OHL concentrations, as low concentrations could unmask subtle phenotypes, while high concentrations might allow us to detect residual induction of strongly defective promoters.

I was especially interested in the six conserved dyad symmetrical sequences (CTG-N_{10}-CAG, positions -8 to +8, see Fig. A.1). Alteration of bases -8, -7, or -6 strongly inhibited the activation of this promoter by CepR, especially at low or intermediate OHL concentrations (Fig. A.1). The same was true of alteration of bases +6, +7, or +8. Alteration of most of the ten central residues (from -5 to +5) inhibited activation at low OHL concentrations but had

less effect at moderate or high concentrations. The $A_{-3}T$ mutation was an exception, as it had little effect on expression at any OHL concentration.

Mutations at positions -9 or -10 caused moderate loss of induced expression, while mutations at +9 or +10 showed more severe defects. One explanation could be that mutations at +9 or +10 might alter sites required for RNA polymerase binding. The *cep* box is centered 44.5 nucleotides upstream of the transcription start site (Weingart *et al.*, 2005), so any -35 motif should lie directly adjacent to the *cep* box. At positions +8 to +13 there is the sequence GTTACA, which resembles the *E. coli* consensus TTGACA at four positions. To learn whether these bases serve as a -35 sequence, I made several mutations to either weaken or strengthen the similarity to the *E. coli* consensus sequence. The mutation $A_{+8}T$ and $T_{+10}G$ were predicted to strengthen the -35 motif. However, neither single mutation nor a double mutation increased the basal or induced expression (Fig. A.1). The mutation $A_{+11}C$ was predicted to decrease expression, but surprisingly, had the opposite effect. An $A_{+13}C$ was also predicted to weaken a -35 sequence, but had only a mild defect. These data strongly suggest that the sequence GTTACA is *not* a functional -35 site. However, the mutations in this region did have fairly strong phenotypes, and may therefore contribute in an undefined way to promoter expression.

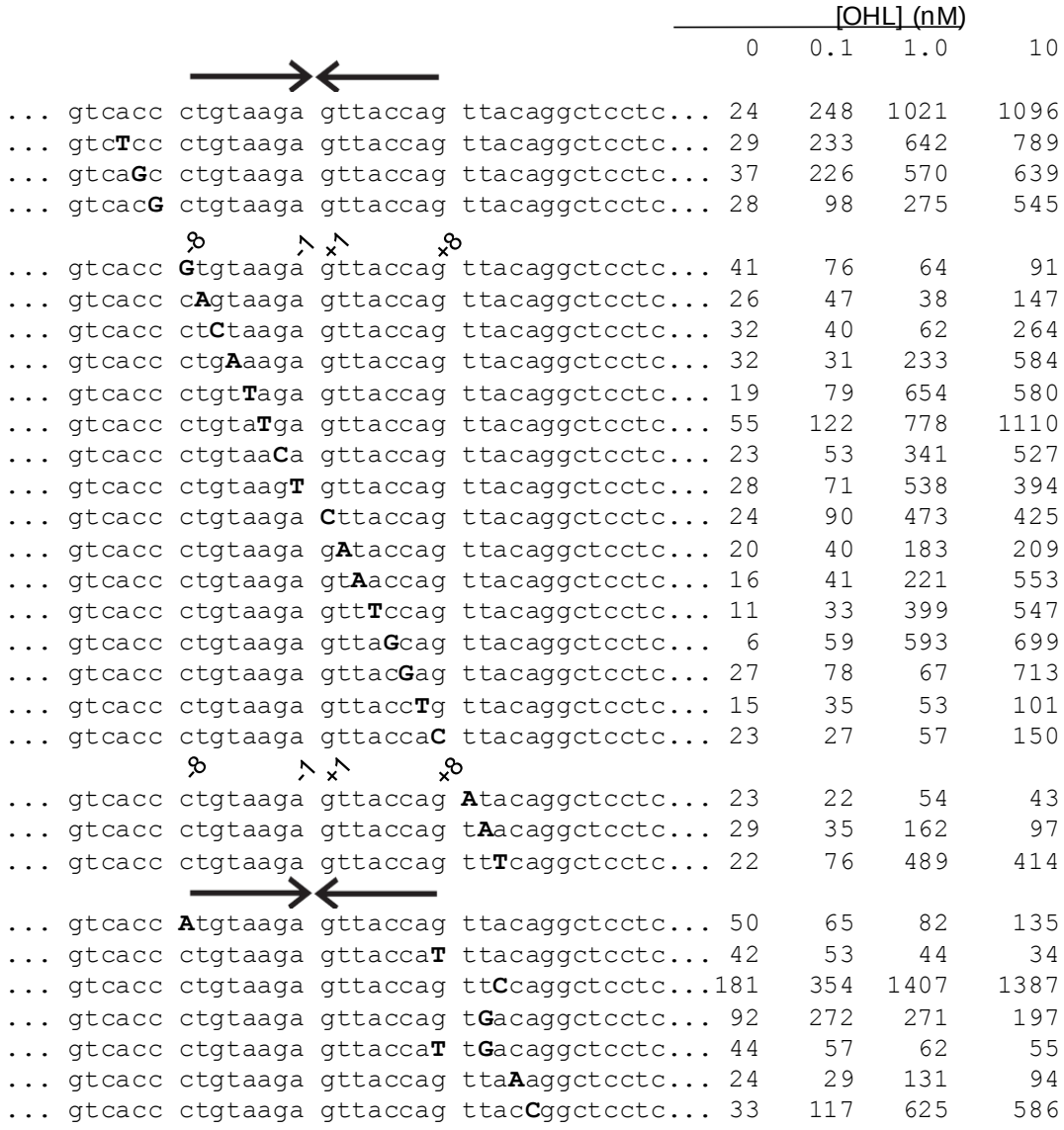

Figure A.1. Mutagenesis of individual nucleotides within and adjacent to the CepR binding site of the *cepI* promoter. Bases are numbered relative to the center of the axis of rotational symmetry, which is indicated using inverted arrows. Fusions were introduced into strain K56 I2, which lacks CepI. Strain K56 and its derivatives are naturally Lac$^-$. Expression of each P*cepI-lacZ* fusion was measured for β-galactosidase specific activity (Miller, 1972) in the presence of the indicated concentrations of OHL.

A.4. Discussion

See the published manuscript[4].

A.5. Experimental Procedures

Note that only the procedures for the experiments that I did are described here. See the published manuscript[4] for the complete experimental procedures for this study.

Bacterial strains and growth conditions

Strains used in this study are described in Table A.1. As needed, *B. cenocepacia* was cultured in 100 µg/ml of trimethoprim or 300 µg/ml of tetracycline. *E. coli* strains were cultured with 15 µg/ml of tetracycline, 100 µg/ml of streptomycin, or 100 µg/ml of ampicillin.

Systematic mutagenesis of the *cepI* promoter

Single-site mutations in the *cepI* promoter were constructed by site-directed mutagenesis using oligonucleotides listed in Table A.2 (IDT, Coralville, Iowa). PCR fragments containing the desired mutations were cloned into the *Kpn*I and *Pst*I sites of pYW302, introduced into *E. coli* strain DH5α by transformation, checked by automated DNA sequencing (Cornell Biotechnology Resource Center), and then introduced into *B. cenocepacia* strain K56 I2 by electroporation (Cangelosi *et al.*, 1991). Transformants were cultured in LB medium at 37^{O} C to mid log phase (OD_{600} of 0.3-0.4), then

diluted 20-fold into LB medium containing 0, 0.1 nM, 1 nM or 1 μM OHL. Cultures were incubated at 37^{O} C with aeration until an OD_{600} of approximately 0.5, and then assayed for β-galactosidase specific activity (Miller, 1972). *B. cenocepacia* is naturally Lac^{-}. Experiments were performed in triplicate with three different isolates of each strain.

Table A.1. Strains and Plasmids

Strains	Relevant features	Reference
K56-I2	*B. cenocepacia* K56 *cepI*::TpR	(Lewenza *et al.*, 1999)
DH5α	*E. coli* α-complementation	Stratagene
Plasmids		
pKP302	Broad host range, promoterless *lacZ* gene, Km^{R}	(Pappas and Winans, 2003)
pYW302	Derivative of pKP302 expressing Tc^{R}	This study
pYW313	Wild type *cepI* promoter cloned into pYWN302	This study
pEC300	Same as pYW313, but with substitution at position -11 (A to T) of the *cep box*, oligonucleotides EDC300 and YWP157	This study
pEC301	Same as pYW313, but with substitution at position. -10 (C to G) of the *cep box*, oligonucleotides EDC301 and YWP157	This study
pEC302	Same as pYW313, but with substitution at position -9 (C to G) of the *cep box*, oligonucleotides EDC302 and YWP157	This study
pEC303	Same as pYW313, but with substitution at position -8 (C to G) of the *cep box*, oligonucleotides EDC303 and YWP157	This study
pEC304	Same as pYW313, but with substitution at position -7 (T to A) of the *cep box*, oligonucleotides EDC304 and YWP157	This study
pEC305	Same as pYW313, but with substitution at position -6 (G to C) of the *cep box*, oligonucleotides EDC305 and YWP157	This study
pEC306	Same as pYW313, but with substitution at position -5 (T to A) of the *cep box*, oligonucleotides EDC306 and YWP157	This study
pEC307	Same as pYW313, but with substitution at position -4 (A to T) of the *cep box*, oligonucleotides EDC307 and YWP157	This study
pEC308	Same as pYW313, but with substitution at position -3 (A to T) of the *cep box*, oligonucleotides EDC308 and YWP157	This study
pEC309	Same as pYW313, but with substitution at position -2 (G to C) of the *cep box*, oligonucleotides EDC309 and YWP157	This study

Table A.1. (Continued)

pEC310	Same as pYW313, but with substitution at position -1 (A to T) of the of the *cep box*, oligonucleotides EDC310 and YWP157	This study
pEC311	Same as pYW313, but with substitution at position +1 (G to C) of the *cep box*, oligonucleotides EDC311 and YWP157	This study
pEC312	Same as pYW313, but with substitution at position +2 (T to A) of the *cep box*, oligonucleotides EDC312 and YWP157	This study
pEC313	Same as pYW313, but with substitution at position +3 (T to A) of the *cep box*, oligonucleotides EDC313 and YWP157	This study
pEC314	Same as pYW313, but with substitution at position +4 (A to T) of the *cep box*, oligonucleotides EDC314 and YWP157	This study
pEC315	Same as pYW313, but with substitution at position +5 (C to G) of the *cep box*, oligonucleotides EDC315 and YWP157	This study
pEC316	Same as pYW313, but with substitution at position +6 (C to G) of the *cep box*, oligonucleotides EDC316 and YWP157	This study
pEC317	Same as pYW313, but with substitution at position +7 (A to T) of the *cep box*, oligonucleotides EDC317 and YWP157	This study
pEC318	Same as pYW313, but with substitution at position +8 (G to C) of the *cep box*, oligonucleotides EDC318 and YWP157	This study
pEC319	Same as pYW313, but with substitution at position +9 (T to A) of the *cep box*, oligonucleotides EDC319 and YWP157	This study
pEC320	Same as pYW313, but with substitution at position +10 (T to A) of the *cep box*, oligonucleotides EDC320 and YWP157	This study
pEC321	Same as pYW313, but with substitution at position +11 (A to T) of the *cep box*, oligonucleotides EDC321 and YWP157	This study
pEC322	Same as pYW313, but with substitution at position -8 (C to A) of the *cep box*, oligonucleotides YWP258 and YWP157	This study
pEC323	Same as pYW313, but with substitution at position +8 (G to T) of the *cep box*, oligonucleotides YWP255 and YWP157	This study
pEC324	Same as pYW313, but with substitution at position +11 (A to C) of the *cep box*, oligonucleotides EDC322 and YWP157	This study
pEC325	Same as pYW313, but with substitution at position +10 (T to G)of the *cep box*, oligonucleotides YWP256 and YWP157	This study
pEC326	Same as pYW313, but with substitution at positions +8 (G to T) and +10 (T to G) of the *cep box*, oligonucleotides YWP257 and YWP157	This study
pEC327	Same as pYW313, but with substitution at position +12 (C to A) of the *cep box*, oligonucleotides EDC323 and YWP157	This study
pEC328	Same as pYW313, but with substitution at position +13 (A to C) of the *cep box*, oligonucleotides EDC324 and YWP157	This study

Table A2. Oligonucleotides.

Name	Sequence	Comments
EDC300	GCTGGTACCACGCCGTC**T**CCCTGTAAGAGTTACCAG	For generating -11T mutation
EDC301	GCTGGTACCACGCCGTCA**G**CCTGTAAGAGTTACCAG	For generating -10G mutation
EDC302	GCTGGTACCACGCCGTCAC**G**CTGTAAGAGTTACCAG	For generating -9G mutation
EDC303	GCTGGTACCACGCCGTCACC**G**TGTAAGAGTTACCAG	For generating −8G mutation
EDC304	GCTGGTACCACGCCGTCACCC**A**GTAAGAGTTACCAGTTACAG	For generating -7A mutation
EDC305	GCTGGTACCACGCCGTCACCCT**C**TAAGAGTTACCAGTTACAG	For generating -6C mutation
EDC306	GCTGGTACCACGCCGTCACCCTG**A**AAGAGTTACCAG	For generating -5A mutation
EDC307	GCTGGTACCACGCCGTCACCCTGT**T**AGAGTTACCAG	For generating -4T mutation
EDC308	GCTGGTACCACGCCGTCACCCTGTA**T**GAGTTACCAG	For generating -3T mutation
EDC309	GCTGGTACCACGCCGTCACCCTGTAA**C**AGTTACCAGT	For generating -2C mutation
EDC310	GCTGGTACCACGCCGTCACCCTGTAAG**T**GTTACCAGT	For generating -1T mutation
EDC311	GCTGGTACCACGCCGTCACCCTGTAAGA**C**TTACCAGT	For generating +1C mutation
EDC312	GCTGGTACCACGCCGTCACCCTGTAAGAG**A**TACCAGT	For generating +2A mutation
EDC313	GCTGGTACCACGCCGTCACCCTGTAAGAGT**A**ACCAGTTAC	For generating +3A mutation
EDC314	GCTGGTACCACGCCGTCACCCTGTAAGAGTT**T**CCAGTTAC	For generating +4T mutation
EDC315	GCTGGTACCACGCCGTCACCCTGTAAGAGTTA**G**CAGTTAC	For generating +5G mutation

Table A.2. (Continued)

EDC316	GCTGGTACCACGCCGTCACCCTGTAAGAGTTAC**G**AGTTACAGGCTCCTCGT	For generating +6G mutation
EDC317	GCTGGTACCACGCCGTCACCCTGTAAGAGTTACC**T**GTTACAGGCTCCTCGTG	For generating +7T mutation
EDC318	GCTGGTACCACGCCGTCACCCTGTAAGAGTTACCA**C**TTACAGGCTC	For generating +8C mutation
EDC319	GCTGGTACCACGCCGTCACCCTGTAAGAGTTACCAG**A**TACAGGCTC	For generating +9A mutation
EDC320	GCTGGTACCACGCCGTCACCCTGTAAGAGTTACCAGT**A**ACAGGCTC	For generating +10A mutation
EDC321	GCTGGTACCACGCCGTCACCCTGTAAGAGTTACCAGTT**T**CAGGCTC	For generating +11T mutation
YWP258	GGGGTACCACGCCGTCACC**A**TGTAAGAGTTACCAGTTACAG	For generating -8A mutation
YWP255	GGGGTACCACGCCGTCACCCTGTAAGAGTTACCA**T**TTACAGGCTCCTCGTGC	For generating +8T mutation
EDC322	GCTGGTACCACGCCGTCACCCTGTAAGAGTTACCAGTT**C**CAGGCTC	For generating +11C mutation
YWP256	GGGGTACCACGCCGTCACCCTGTAAGAGTTACCAGT**G**ACAGGCTCCTCGTGCCG	For generating +10G mutation

Table A.2. (Continued)

YWP257	GGGGTACCACGCCGTCACCCTGTAAGAGTTACCA**T**T**G**ACAGGCTCCTCGTGCCG	For generating +8T +10G mutation
EDC323	GCTGGTACCACGCCGTCACCCTGTAAGAGTTACCAGTTA**A**AGGCTC	For generating +12A mutation
EDC324	GCTGGTACCACGCCGTCACCCTGTAAGAGTTACCAGTTAC**C**GGCTC	For generating +13C mutation
YWP157	AACTGCAGGCCGATAGCGCCCGAGATCC	Reverse primer for PCR amplifying mutant *cepI* promoters

A.6. References

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